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Application of cinchona-sulfonate-based chiral zwitterionic ion exchangers for the separation of proline-containing dipeptide rotamers and determination of on-column isomerization parameters from dynamic elution profiles

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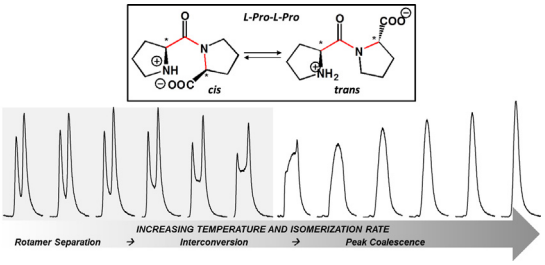
HIGHLIGHTS

- X-Pro peptides produce interconverting elution profiles on zwitterionic stationary phases.
- The *cis–trans* isomerization rate in solution is lower than in the chromatographic system.
- Cinchona-based zwitterionic selectors apparently lower the isomerization barrier.
- Rotamer separation is feasible <0 °C, enantiomer separation above room temperature.

GRAPHICAL ABSTRACT



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ABSTRACT

The interconversion of *cis* and *trans* isomers of dipeptides containing *C*-terminal proline was studied by dynamic chromatography on zwitterionic chiral stationary phases at temperatures ranging from −15 °C to +45 °C The *cis–trans* isomers could be separated below 0 °C and above 0–10 °C plateau formation and peak coalescence phenomena occurred, which is characteristic for a dynamic process at the time-scale of partitioning. At and above room temperature, full coalescence was observed, which allowed separations of enantiomers without interference from interconversion effects. Analysis of the dynamic elution profiles of the interconverting peptides allowed the determination of isomerization rate constants and thermodynamic activation parameters (isomerization enthalpy, entropy and activation energy). In accordance with established results, isomerization rates and thermodynamic parameters were found to depend on the nature of the *N*-terminal amino acid. Isomerization barriers were only slightly lower than values determined with other methods but significant differences in the relative contributions of the activation enthalpy and entropy as well as isomerization rates pointed toward selector-moderated isomerization dynamics.

Abbreviations: AX, anion exchange(r); CAD, charged aerosol detector; (C)SP, (chiral) stationary phase; C(Z)E, capillary (zone) electrophoresis; HILIC, hydrophilic interaction liquid chromatography; IX, ion exchange(r); NMR, nuclear magnetic resonance; RP, reversed phase; QN, quinine; QD, quinidine; Tau-QN, "Taurine-Quinine" (ethanesulfonic acid-carbamoylated quinine); ZWIX, zwitterion exchange(r).

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1. Introduction

Chiral, zwitterionic ion exchanger stationary phases (ZWIX-type CSPs) such as ethanesulfonic acid-quinine ("Tau-QN", CSP 1 in Fig. 1)-modified silica were developed for enantiomer separations of chiral amphoteric compounds (selectands), for instance free amino acids and peptides [1–3].

On ZWIX CSPs, the analytes are retained by simultaneous double ion pairing between their acidic and basic termini and positively and negatively charged groups of the semi-synthetic selectors. Hydrogen bond formation, dipole-dipole interaction, aromatic (π–π stacking), steric and solvophobic interactions take place in a concerted fashion between the respective functional groups of selectors and selectands and lead to distinction and, consequently, chromatographic separation of the analyte stereoisomers. Directed and undirected as well as repulsive and attractive recognition increments can contribute to the distinction of isomeric solutes. Recently, a study concerning sequence stereoisomers of small, synthetic oligo-phenylalanine (Phe) peptides (e.g. D,L,L-Phe-3, L,D,L-Phe-3 and L,L,D-Phe-3) demonstrated the extension of the application spectrum of ZWIX CSPs to separations of non-enantiomeric stereoisomers (i.e. epimers and positional stereoisomers) [4].

All previously reported peptide stereoisomer separations using ZWIX CSPs concerned peptides comprised exclusively of primary amino acids. However, it is well known that secondary amino acids, in particular proline, play a key role for higher-order structures of peptides and proteins and, consequently, their biological activity [5–7].

A peptide (amide) C—N bond is essentially planar due to its partial π-character. *Cis* and *trans* isomers (conformers) of a peptide are related by a 180° rotation around this bond (rotamerism). The conformers of some dipeptides containing *C*-terminal proline are depicted in Fig. 2.

Peptide bonds containing *C*-terminal proline (denoted X-Pro, with X representing a generic amino acid; here the abbreviation "X-Pro" refers to unprotected dipeptides) are rotationally hindered with an interconversion barrier $\Delta G^{\ddagger}$ of about 20 kcal (83.7 kJ mol^{-1}) [8,9]. They are much more likely in the *cis* conformation than peptide bonds between primary amino acids due to stabilization by intramolecular hydrogen bonds. In addition, the *cis* and *trans* forms of X-Pro peptides suffer from similar steric hindrance on account of the substituted (secondary) amide (Fig. 1). Consequently, up to 40% of *cis*-configured peptide bonds are frequently found in such peptides as opposed to only 1% cis between primary amino acids [10,11].

Oligo-proline regions in peptides and proteins form polyproline helices whose sense of rotation depends on the conformation of the involved residues. These polyproline motifs are thought to play a role in signal transduction pathways [12–14]. Interestingly, *cis*-configured substructures are particularly stable to enzymatic cleavage with proteases such as trypsin being capable of cleaving X-Pro bonds (e.g. Lys-Pro or Arg-Pro) in *trans* conformation only [15,16].

The *cis–trans* isomerization of X-Pro peptide bonds in vivo, where the peptide is embedded in a polypeptide or protein sequence, is catalyzed by prolyl isomerases and believed to play an important role in protein folding [11,17–20]. The spontaneous conversion in vitro is very slow, with rate constants as low as 10^{-2}–10^{-3} s^{-1} at 298 K [21]. This allows chromatographic [21–23] or electrophoretic separations [24–29] of the isomers at suitably low temperatures.

In chromatographic experiments, the *cis–trans* isomerization kinetics of proline-containing peptides and the respective equilibrium constants can vary depending on the type of solvent [22,30], the charge state of the peptide [11] and the amino acid sequence, i.e. the number and nature of neighboring amino acid residues [31,32].

In addition, the stationary phase of the chromatographic system or the capillary wall of an electrophoretic system must be taken into consideration as it can affect the interconversion. This was demonstrated by capillary zone electrophoresis (CZE) studies on X-Pro isomerization during which a catalytic effect of the fused-silica capillary wall on the isomerization was discovered [30]. Observations made in reversed-phase (RP) chromatography experiments also pointed toward an influence of the stationary phase on the interconversion dynamics [21,33].

Chiral stationary phases demand special consideration in this context because they can be envisaged to change the kinetics [34], but possibly also the thermodynamic equilibrium of an interconversion reaction compared to an achiral environment. For example, Trapp and Schurig demonstrated the effect of a chiral stationary phase on the enantiomerization barrier of Tröger's base [35]. Maier et al. developed a three-column HPLC approach to investigate stationary phase and solvent effects in great detail [36].

Assuming a stationary phase with sufficient selectivity for the *cis*/*trans* isomers, three different combinations of the

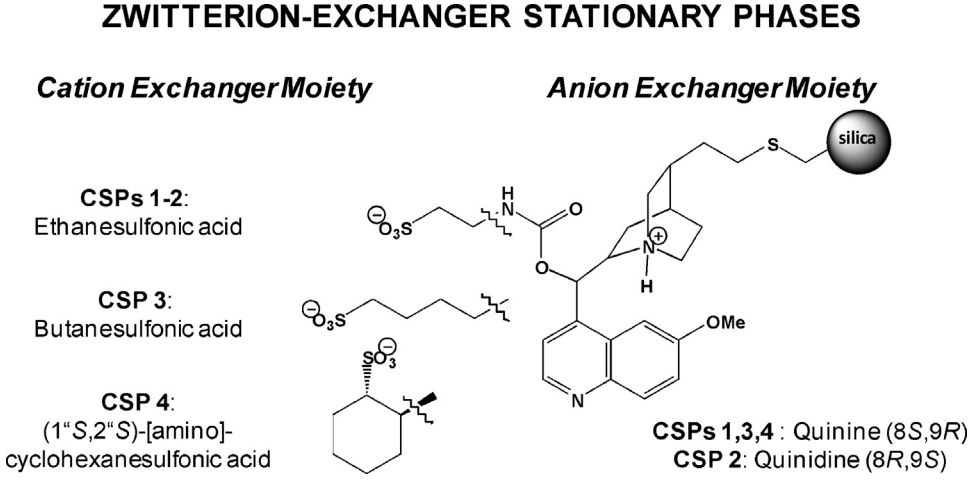


Fig. 1. Zwitterionic chiral stationary phases prepared by fusion of strong cation exchanger and weak anion exchanger motifs. CSPs 1 and 2 are derived from achiral taurine (aminoethanesulfonic acid) and feature pseudo-enantiomeric anion exchanger motifs. CSPs 1, 3 and 4 are based on quinine and bear carbamoyl substituents with different structural characteristics.

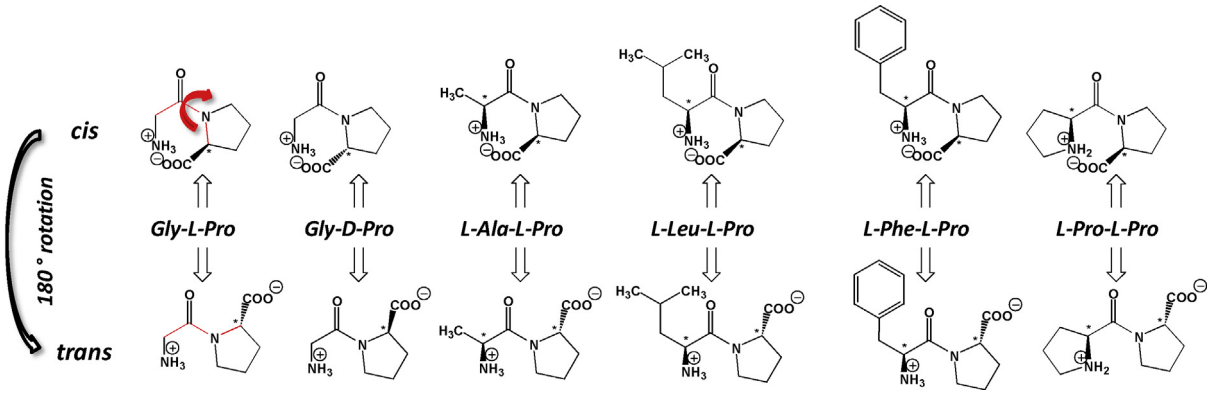


Fig. 2. *Cis* and *trans* isomers (rotamers) of peptides containing C-terminal proline.

chromatographic separation and cis–trans interconversion of the proline-containing peptides are conceivable [37]: (a) The interconversion is slow compared to the separation process, in which case two distinct and fully separated peaks are observed for the two conformers. (b) The interconversion is significantly faster than the chromatographic separation and a single peak representing a mixture of all isomerization states is detected. (c) The interconversion and the separation occur at similar time scales and the two peaks containing the two conformers are connected by a plateau which represents the intermediate stages of the interconversion.

Temperature variation strongly influences the isomerization rate and can be used to shift the system toward either of these scenarios. The chromatographic parameters of the dynamic peak profiles can be used to determine kinetic interconversion rates by data fitting procedures or with dedicated software (see below) [37–41].

This study aimed at (i) exploring the discriminating potential of ZWIX CSPs toward *cis* and *trans*-configured rotational isomers of free X-Pro peptides and (ii) the investigation of the dynamics of on-column isomerization processes using DCXplorer, a software based on the unified equation of dynamic chromatography [42]. This software allows for the calculation of kinetic rate constants k_1 (forward reaction) and k_{-1} (backward reaction) from the relative heights of the interconverting peaks, the plateau between them and the respective retention times of the isomers. The calculations are based on time-dependent distribution functions derived from the stochastic model of chromatography [43]. Thermodynamic activation enthalpy ($\Delta H^{\ddagger}$) and entropy ($\Delta S^{\ddagger}$) as well as the activation energy $\Delta G^{\ddagger}$ can subsequently be obtained using the Eyring equation and plots of $\ln(k_1 \cdot T^{-1})$ vs. T^{-1} (Eyring plots). Their evaluation provides the basis for a discussion on the influences of the chromatographic system, in particular the chiral stationary phase, and the *N*-terminal amino acid of X-Pro dipeptides on the on-column isomerization phenomena.

While such experiments can greatly enhance the understanding of molecular interactions, one has to bear in mind that the reaction rates and thermodynamic data obtained represent weighted averages of the processes occurring in the mobile and stationary phases. Unequivocal identification and quantification of effects of the stationary phase are not feasible, and neither is the isolation of the contributions of the chiral selector. Nonetheless, as will be demonstrated in the following sections, the obtained data provide new insights into the recognition potential of *Cinchona*-sulfonate-based ZWIX CSPs (see Fig. 1). In addition, they facilitate cautious comparison of the isomerization dynamics of X-Pro peptides on ZWIX CSPs with their behavior in solution or in other chromatographic systems.

2. Experimental

2.1. Materials

Gradient grade methanol and acetonitrile were purchased from Sigma–Aldrich Austria (Vienna, Austria) and Merck (Darmstadt, Germany). Ultra-pure water was obtained from an in-house Millipore facility (Millipore GmbH, Vienna, Austria). Formic acid, acetic acid, ammonium hydroxide, diethylamine and ammonium acetate (of analytical grade or higher purity) were from Fluka/Sigma–Aldrich.

Pro-Pro·HCl, Phe-Pro, Leu-Pro·HCl, Arg-Pro (sulfate salt) and Pro-Arg (acetate salt) were commercially available (Bachem, Bubendorf, Switzerland). Pro-3, Pro-4, Gly-Gly-Pro and Gly-Gly-D-Pro were custom-synthesized by GenScript, Piscataway, NJ, USA. D-Pro-D-Pro, D-Pro-L-Pro, L-Pro-D-Pro, D-Pro-3 and D-Pro-4 were prepared by PiChem (Graz, Austria) and Gly-D-Pro and D-Pro-Gly were from GeneCust Europe (Dudelange, Luxembourg). The peptides were of >90% purity according to manufacturer specifications. Other peptides were kind gifts from research partners (purity not specified).

3. Methods

3.1. HPLC

Chromatographic experiments were performed on an Agilent 1200 Rapid Resolution HPLC system (Agilent, Waldbronn, Germany) equipped with binary pump, cooled autosampler (4 °C) and thermostated column compartment. For detection, a Corona Charged Aerosol detector (Dionex/Thermo Fisher Scientific, Sunnyvale, CA, USA) was employed. Void times were determined by injection of 10 μL of toluene/methanol (1:1). Peptides were dissolved in the mobile phase at concentrations of 1 mg mL^{-1} (qualitative measurements) or 5 μmol mL^{-1} (determination of rate constants) and the samples were agitated at room temperature (600 rpm, 15 min) prior to each injection. Injection volumes for peptide measurements were 3 μL. Chromatographic experiments were conducted under isocratic conditions with methanol (50 mM formic acid, 25 mM diethylamine) as the mobile phase. Flow rates were 0.4 mL min^{-1} for columns with 3 mm i.d. and 1 mL min^{-1} for columns with 4 resp. 4.6 mm i.d. unless otherwise specified.

The column temperature was controlled by an F34-ME refrigerated/heating circulator (Julabo, Seelbach, Germany) filled with an ethylene glycol-based heating/cooling medium into which the analytical column and the connecting steel capillaries were immersed. In order to prevent a temperature gradient along the chromatographic system, the mobile phase was pre-cooled (down to 4 °C)

for all experiments below 25 °C and pre-thermostated by flowing through the heat exchanger (column compartment) of the HPLC system that was set accordingly between 8 °C, the lowest temperature obtainable, and 40 °C. After the system reached the desired temperature, the column was equilibrated for at least 30 more minutes.

The material for CSP 1 "Tau-QN" (3 μm spherical silica, pore size: 120 Å, surface area: 300 $m^2\,g^{-1}$, selector coverage: 174 $\mu mol\,g^{-1}$) was prepared in accordance with published procedures [1] and slurry-packed into a stainless steel column (150 × 3 mm i.d.) in-house. The synthetic procedures toward CSPs 2–4 are reported elsewhere [1,2]. CHIRALPAK ZWIX (+) (CSP 4 in Fig. 1) is now commercially available from Chiral Technologies (Illkirch, France). Column dimensions of CSPs 2 and 3 were 150 × 4 mm i.d. (5 μm material, selector coverage 150 resp. 254 $\mu mol\,g^{-1}$), CSP 4 was a 250 × 3 mm column (coverage: 233 $\mu mol\,g^{-1}$).

For the reversed-phase control experiment, a Kinetex C-18 column (100 × 3 mm i.d., 2.6 μm material) from Phenomenex (Aschaffenburg, Germany) was employed. The non-chiral zwitterionic stationary phase was a Nucleodur HILIC 100-3 column (150 × 4.6 mm i.d., 3 μm material) from Macherey-Nagel (Düren, Germany).

All HPLC experiments were repeated once after several weeks to ensure reproducibility. The results reported in this paper deviate from the control experiment by less than 2%.

3.2. Software

Agilent ChemStation software (Version Rev. B.04.03) was used for HPLC data acquisition and processing. Isomerization rate constants were determined with DCXplorer Software, Version 1.0.0.9 [43]. ACD/Labs software (Version 7.0) was used to calculate pK_a values. Data evaluation was carried out using Microsoft Excel.

3.3. Determination of kinetic and thermodynamic data from chromatograms

Isomerization rate constants k_1 and k_{-1} (in order to avoid confusion, chromatographic retention factors are distinguished with the letter "R" as a subscript: k_R) were calculated from the interconverting elution profiles of X-Pro peptides at temperatures between −15 °C and +15 °C using the DCXplorer software (the upper limit of the temperature range in which interconversion could be observed varied for each peptide). Here the elution profile is analyzed by the software and parameters fully characterizing the chromatographic peak-shapes are used in the unified equation of chromatography to determine reaction rate constants with high precision. Due to the experimental setup, peak assignment was not feasible. Hence, the two isomer peaks were arbitrarily assigned A (first eluting isomer) and B (second eluting isomer). Note that due to the non-enantiomeric nature of the isomers, inconsistent elution orders (*cis* before *trans* resp. *trans* before *cis*) are theoretically conceivable for the individual peptides.

The activation parameters $\Delta S^{\ddagger}$ and $\Delta H^{\ddagger}$ for the conversion of A to B were determined from the slope and intercept of plots of $\ln(k_1 \cdot T^{-1})$ vs. T^{-1} (Eyring plots, Eq. (1)).

$$\ln\frac{k_1}{T} = \frac{-\Delta H^{\ddagger}}{R}\cdot\frac{1}{T} + \ln\frac{k_B}{h} + \frac{\Delta S^{\ddagger}}{R} \quad (1)$$

R represents the universal gas constant (8.314462 $J\,mol^{-1}\,K^{-1}$), k_B the Boltzmann constant ($1.3806488 \times 10^{-23}\,J\,K^{-1}$), h the Planck constant ($6.62606957 \times 10^{-34}\,J\,s$) and T the absolute temperature in Kelvin.

The Gibbs free energy $\Delta G^{\ddagger}$ at temperature T [K] can be obtained using k_1, k_B and h (Eq. (2)):

$$\Delta G^{\ddagger}(T) = -RT\ln\frac{k_1 h}{k_B T}. \quad (2)$$

4. Results and discussion

4.1. Isomerization of proline dipeptides on zwitterionic chiral stationary phases

The brush-type zwitterionic chiral stationary phases (ZWIX CSPs, Fig. 1) used in this study contain low-molecular weight selectors whose chiral weak anion exchanger (WAX, a protonated tertiary amine) and strong cation exchanger (SCX, alkanesulfonic acid) scaffolds are fused via a carbamate bond. They have been shown to facilitate enantiomer separations of zwitterionic compounds such as free amino acids and peptides based on a retention mechanism utilizing, among other increments, simultaneous double ion pairing of the acidic and basic sites of the interacting species [1,2].

A recent contribution from our group demonstrated the recognition potential of ZWIX CSPs for non-enantiomeric (i.e. epimeric and diastereomeric) sequence stereoisomers of oligopeptides comprised of primary amino acids [4]. As an extension of these studies we attempted chiral separations of small peptides containing the secondary amino acid proline, which proved particularly challenging on account of their conformational peculiarities. Unless stated otherwise, all experiments were conducted using CSP 1. Information on the chromatographic behavior of X-Pro peptides on other *Cinchona*-sulfonate-based ZWIX CSPs can be found in the supplementary material.

Addressing the chromatographic separations of diproline (Pro-Pro) stereoisomers, limited column efficiency and asymmetric (tailing) peaks were anticipated based on previous experimental findings regarding peptide separations on ZWIX CSPs. Nonetheless, the abnormal width (up to several minutes) of the peaks produced by the peptides on CSP 1 and especially their distorted elution profiles were startling. The chromatograms were indicative of on-column interconversion of the analytes, which was an unprecedented observation in the context of *Cinchona*-based ZWIX CSPs.

The homochiral dipeptide pair L-Pro-L-Pro and D-Pro-D-Pro (enantiomeric to each other) showed more pronouncedly dynamic elution profiles than their heterochiral stereoisomers L-Pro-D-Pro and D-Pro-L-Pro (the other pair of enantiomers). The phenomenon intensified when the experimental temperature was lowered from 25 °C to 15 °C ("Batman peaks", Fig. 3). Increasing the temperature to 45 °C led to fully coalescent, albeit still broad single peaks. This may suggest either a strongly increasing isomerization rate or decreasing chromatographic selectivity at elevated temperatures. Stereoisomeric impurities and configurational isomerism (i.e. inversion of one or both stereocenters) could be dismissed as reasons for the observed irregularities by comparison of the chromatograms of all four stereoisomers of Pro-Pro. Mass spectrometric control experiments (HPLC-ESI-MS, data not shown) ruled out disintegration of the analytes and confirmed the dynamic peaks as originating from the un-protected Pro-Pro peptides. Chromatograms of tri- and tetraproline also hinted at interconversion processes occurring at the same time scale as the separation. However, the stationary phases employed in our studies did not provide sufficiently high selectivity and efficiency for separations of 4 (Pro-Pro-Pro with 2 X-Pro bonds, each of which can assume either *cis* or *trans* conformation → $2^2 = 4$) or even 8 (Pro-Pro-Pro-Pro, 3 peptide bonds → $2^3 = 8$) conformational isomers. Hence, the discussion provided in this contribution is limited to dimers.

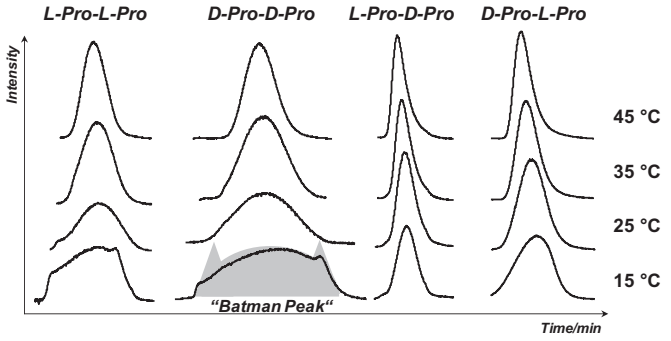


Fig. 3. Irregular elution profiles for diastereomers of Pro-Pro on a zwitterionic chiral stationary phase. For homochiral (L,L and D,D) peptides, slow interconversion is observed at lower temperatures while the isomer peaks coalesce close to room temperature. Heterochiral peptides (L,D and D,L) show a significantly lower temperature-dependence of their interconversion. Chromatographic conditions: CSP 1, 150 × 4 mm i.d., 5 μm material, selector coverage: 219 μmol g^{-1} silica. Mobile phase: methanol, 50 mM formic acid, 25 mM diethylamine, 0.7 mL min^{-1}. Detection: CAD.

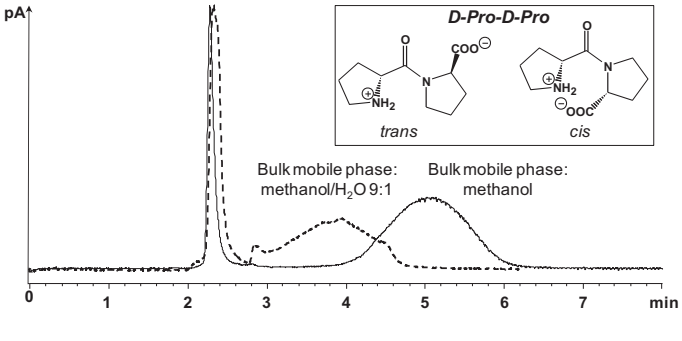


Fig. 4. Increased peak distortion due to slower isomerization of D-Pro-D-Pro in a mobile phase containing 10% of water. Chromatographic conditions: CSP 1, 150 × 4 mm i.d., 5 μm material, selector coverage: 219 μmol g^{-1} silica. Standard mobile phase: methanol, 50 mM formic acid, 25 mM diethylamine, Aqueous mobile phase: methanol (50 mM formic acid, 25 mM diethylamine)/water (9:1, v/v), 0.7 mL min^{-1}, 25 °C. Detection: CAD.

The interconverting elution profiles are caused by the superposition of a relatively slow *cis–trans* isomerization of the peptide (amide) bond and the chromatographic separation process [37]. At temperatures below 20 °C, the isomerization is slow compared to the separation and the chiral stationary phase is capable of distinguishing the conformers. The fact that heterochiral peptides show less pronouncedly dynamic peak profiles might be ascribed to a lower isomerization barrier compared to homochiral ones [44]. Peptides containing exclusively *N*-terminal proline, e.g. Pro-Phe, exhibited no such behavior under the given experimental conditions (see supplementary material).

It is obvious that the mobile phase must be included in the rationalization of on-column isomerization processes. This is especially relevant for amphoteric solutes such as the free dipeptides (H-X-Pro-OH) which are the subjects of this study because their charge state is defined by their environment.

The chromatographic experiments discussed in this contribution were conducted in polar-ionic mobile phase mode (methanol, acid-to-base ratio 2:1) at an apparent pH of 5–6. Under these conditions, the zwitterionic form of the peptides is prevalent (X-Pro: $pK_a(1)$ = ca. 3.2, $pK_a(2)$ = ca. 7.9–9.2). It has been established that the cationic form of X-Pro peptides favors the *trans* configuration due to a stabilizing H-bond between the protonated *C*-terminus and the amide carbonyl group [45]. Under more basic conditions, when the *C*-terminus is deprotonated, electrostatic interactions between the carboxylate and the protonated *N*-terminus (zwitterionic peptide) as well as the carbonyl oxygen of the peptide group (anionic peptide) support the *cis* conformation and account for significantly higher *cis*/*trans* ratios [44]. The interconverting peaks observed for X-Pro peptides on ZWIX CSPs usually showed a roughly equal distribution of the two isomers in terms of relative peak areas, corresponding to NMR studies of Ala-Pro [in aqueous solvents] which report ca. 10% of *cis*-configured Ala-Pro in the cationic peptide, ca. 40% in the zwitterionic form and ca. 55% in the anionic peptide at 25 °C [31,44]. At this point it should be noted that even though the detector response of the charged aerosol detector (CAD) is expected to be identical for the two isomers, errors are likely to arise from asymmetric and/or overlapping peaks when attempting to estimate isomer ratios from chromatograms.

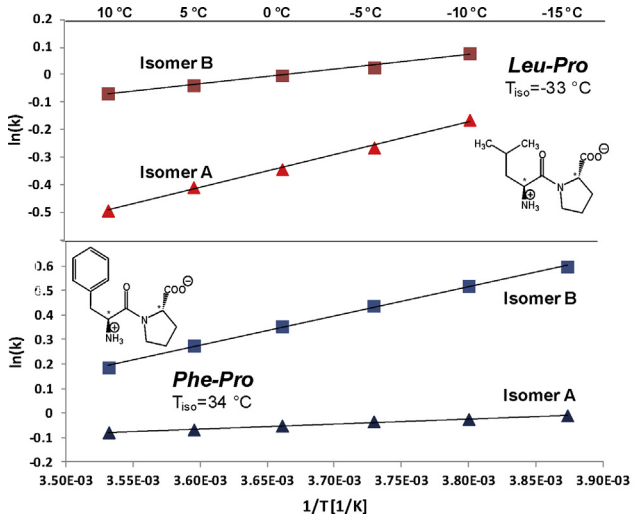
The equilibrium constant *K* of the *cis–trans* isomerization is reported to be only slightly affected by changes in temperature [22] and solvent composition [46]. In contrast to this, the isomerization rate k_1 is (a) known to be strongly temperature-dependent and (b) has been demonstrated to decrease in polar, protic solvents [30,46]; the reason being that the *cis–trans* isomerization of the X-Pro bond requires the disruption of the strong electrostatic interactions between the charged peptide termini and their (re-) solvation in order to reach the transition state. This process is energetically unfavorable in protic solvents such as methanol, and especially in water [19]. Solvent molecules which are capable of forming solvation shells by strong H-bonding inhibit the rotation because the solvation shell must be stripped off during the isomerization, adding disadvantageous energy contributions [47].

In accordance with the anticipated decrease of the isomerization rate in aqueous solvents, decreased retention and more pronounced distortion of the chromatographic peak of Pro-Pro were observed upon addition of 10 v% of water to the originally methanolic mobile phase (Fig. 4). Previous experiments have also shown that retention times for alanine (Ala), valine (Val) and phenylalanine (Phe) oligopeptides on ZWIX CSPs decreased with increasing water content of the mobile phase. For the latter peptides, shorter retention times were attributed to stronger solvation of the ionic interaction sites of selector and peptide in aqueous media. A strong salvation shell interferes with the ionic interactions required for attachment to the selector (S. Wernisch, *diploma thesis*, University of Vienna, 2010; [2]). However, for the peptides not containing a C-terminal proline, the peak-width inevitably decreased with increasing water content of the mobile phase. It is likely that adsorption–desorption kinetics for charged analytes on ZWIX CSPs are faster in water-containing mobile phases due to more efficient displacement processes but the changes in the peak-shape point toward increasingly pronounced (i.e. slower) on-column interconversion.

4.2. Rotamer recognition of peptides featuring different *N*-terminal amino acids

The following discussions deal with the chromatographic behavior of X-Pro dipeptides with different *N*-termini (chemoselectivity of the stationary phase) and *cis–trans* isomerization dynamics (rotamer selectivity) in the stereoselective chromatographic system. Considerations of enantiomer selectivity can be found in the supplementary material. Unless stated otherwise, the stationary phase is CSP 1.

In addition to their recognition capabilities for the stereoisomers, *Cinchona* alkaloids and their derivatives (the zwitterionic selectors) are potent chiral catalysts [48]. They are envisaged to act as molecular "templates" and encourage conformational changes in the peptides which allow for a better, "induced" selector–selectand fit, especially with regard to the charged moieties. In combination with other, weaker non-covalent forces occurring upon attachment, charge-to-charge interactions are very likely to affect both interconversion kinetics and isomerization equilibria.



Peptide	Isomer	Slope	Intercept	R^2	T_{iso} [°C]
Gly-L-Pro	A	571.07	-2.3695	0.9921	-42
	B	216.42	-0.8349	0.9898	
Gly-D-Pro	A	412.69	-1.6500	0.8131	25
	B	1240.40	-4.4254	0.9718	
Ala-Pro	A	833.00	-3.5081	0.9985	-29
	B	255.02	-1.1376	0.9944	
Leu-Pro	A	1199.80	-4.7277	0.9965	-33
	B	531.10	-1.9437	0.9905	
Phe-Pro	A	205.13	-0.8030	0.9937	34
	B	1201.80	-4.0492	0.9983	
Pro-Pro	A	800.93	-3.0247	0.9905	-41
	B	413.03	-1.3518	0.9936	

Fig. 5. Van't Hoff Plots (logarithmic retention factor vs. inverse temperature) for peptides on CSP 1. The calculated iso-stereoselective temperature (i.e. the intersection of the ln *k* plots of isomers A and B), is positive [°C] for Gly-D-Pro and Phe-Pro (lower left panel) and negative for all other peptides, meaning that theoretically the chromatographic selectivity should improve with increasing temperature for most peptides. In reality, this thermodynamic effect is outweighed by the rapid increase in kinetic interconversion rate at higher temperatures.

Although our experimental setup did not facilitate the determination of isomer ratios, the preferred conformation of the peptide adsorbed on the stationary phase is expected to deviate significantly from a peptide in solution. On account of the numerous interactions occurring between the peptide and the selector, the observed kinetic rate constants of interconversion will also change due to the attachment to the selector. In principle, both enhancing and diminishing effects are conceivable. Predictions based on the stereochemical features of the selectands are not feasible but the careful evaluation of chromatographic parameters such as peak-shape, retention factors and selectivity coefficients can provide a framework for discussions regarding the influence of interactions of the selectand and the stationary phase on the isomerization dynamics of different X-Pro peptides. In this discussion no distinction can be made between effects originating from stereoselective and non-stereoselective attachment sites (e.g. end-capping groups).

Small, sterically undemanding *N*-terminal amino acids such as glycine (Gly) have been shown to enhance the kinetic isomerization rate of X-Pro peptides compared to more bulky residues such as Phe [11]. Concerning the thermodynamic equilibrium, higher *cis*/*trans* ratios are expected for peptides comprising aromatic *N*-terminal residues than aliphatic ones [44,49–51]. A quick estimation of influences stemming from the CSP was based on the chromatographic behavior of a number of X-Pro dipeptides with varying *N*-termini (X = Gly, Ala, Leu, Phe, Pro) at different temperatures (5 °C steps from −15 °C to +40 °C, see below).

Eq. (3) describes the thermodynamic basis for isomer separation. Isomer selectivity α_{rot} (calculated from the respective retention factors $k_R(A)$ and $k_R(B)$) arises from a difference in the Gibbs energies of selector–selectand complex formation. α is temperature-dependent, meaning that increase of the temperature can result in either an increase or a decrease of selectivity, depending on the analyte-specific iso-[rotamer]-selective temperature at which $\Delta\Delta G = 0$ [52]. This temperature can be determined from plots of ln k_R against the inverse temperature (van't Hoff plots, see below).

$$\Delta\Delta G^0_{A,B} = \Delta G^0_A - \Delta G^0_B = -RT \ln\frac{K_1}{K_2} = -RT \ln\alpha,$$

$$\text{with } \alpha = \frac{k_R(B)}{k_R(A)} \qquad (3)$$

For all peptides, two distinct peaks were observed at temperatures below 0 °C. In the case of Phe-Pro, the two isomers were fully baseline separated at −15 °C and the rotamer selectivity coefficient α_{rot} decreased as the temperature increased. For all other peptides, the numerical value of the selectivity coefficient increased with temperature before peak coalescence occurred due to accelerating interconversion. In particular, we observed the occurrence of plateaus, "Batman" peaks and, eventually, peak coalescence above 5–15 °C. The coalescent peaks of the other peptides were still broader than those of Phe-Pro, which reflects the finding that the rotamer selectivity for the latter peptide decreased with increasing temperature.

Van't Hoff plots (ln k_R vs. T^{-1}) translated the increasing rotamer selectivity for Gly-Pro, Ala-Pro, Leu-Pro and Pro-Pro into iso-[rotamer]selective temperatures (intersection of the plots for isomers A and B) below −15 °C, meaning that our experiments were conducted at temperatures which theoretically promote rotamer selectivity. The decreasing selectivity in the case of Phe-Pro resulted in T_{iso} = 34 °C (Fig. 5). Interestingly, a positive value for T_{iso} was also determined for Gly-D-Pro (see supplementary information). However, our observations did not reflect the calculated values very well because plateau formation and mediocre resolution resulted in effective peak coalescence below room temperature for all peptides. Besides, the selectivity was calculated from the retention times of the peak maxima without consideration of the asymmetry (fronting and tailing, respectively) of the chromatographic peaks. Therefore, the α_{rot} numbers are a simplified and not fully accurate representation of the chromatographic results. The retention times of the individual peptide isomers can be found in the supplementary material.

The temperature range of our experiments encompassed the whole range of conceivable interconversion/separation scenarios: The rotamers interconvert slowly and are well recognized by the CSP at low temperatures but as the temperature is increased, the (numerically) increasing rotamer selectivity of the stationary phase for most peptides is outweighed by the enhancement of interconversion phenomena due to the amplification of the kinetic isomerization rate. At temperatures above 15 °C, the interconversion is fast compared to the chromatographic processes and full coalescence is observed. Fig. 6 shows the chromatograms obtained for Pro-Pro on CSP 1.

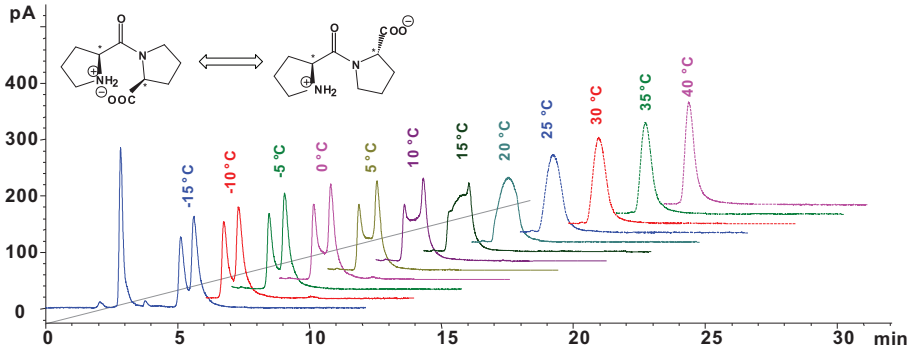


Fig. 6. Chromatograms showing the behavior of L-Pro-L-Pro on CSP 1 over a temperature range of −15 °C to +40 °C. Mobile phase: methanol, 50 mM formic acid, 25 mM diethylamine, 0.4 mL min^{-1}. Injection peaks were removed for clarity.

Generally, the higher retention factors of Phe-Pro indicate a higher degree of selector–selectand interaction compared to less strongly retained peptides. In return, the selector is likely to induce conformational changes in Phe-Pro, meaning that the isomerization parameters of this peptide are expected to be controlled by the CSP to a higher degree than those of the remaining peptides.

The peak area ratios of isomers A (first eluting isomer) and B (second eluting isomer) were observed to shift with increasing temperature but drawing at peak area ratios to determine the position of the *cis–trans* equilibrium is not advisable when dealing with tailing peaks. To fully elucidate the influence of the chiral selector on the equilibrium, the results would have to be interpreted with regard to the chromatographic phase ratio as well as the presence/absence of non-stereoselective adsorption sites of the CSP. The experiments required for this evaluation, however, were outside of the scope of this study.

As the chromatographic results allowed only for educated guesses regarding the dynamic phenomena occurring on the level of interatomic bonds, we are not yet able to provide a full mechanistic explanation for our chromatographic findings. A major issue was that the analyte-specific interaction increments facilitating stereoselective separations on our ZWIX-type CSPs prevented the prediction of elution orders, a feat that *has* been accomplished by Kalmán et al. for the retention of X-Pro *cis–trans* isomers in non-chiral reversed-phase (RP) HPLC experiments [22]. Due to the non-stereospecific nature of the CAD detector, assignment of the rotamer peaks to the respective isomers was not feasible. Considering the nature of a chiral (i.e., essentially *enantio*selective) stationary phase, one must also take into account the possibility of rotamer elution order reversal for enantiomeric peptides (e.g. *cis* before *trans* for Gly-D-Pro and *trans* before *cis* for Gly-L-Pro).

In order to gain a better understanding of the influence of the surface modification on the interconversion dynamics of X-Pro peptides, chromatographic experiments with a non-chiral C-18 and a zwitterionic (HILIC) stationary phase were conducted. The respective concepts of RP and HILIC stationary phases require specific mobile phase conditions which are decidedly different from the mobile phase typically employed in ZWIX studies. The methanol/formic acid/diethylamine system was therefore expected to adversely affect retention of the analytes on these columns. Nonetheless, the polar-organic ionic mobile phase was maintained in order not to blend in effects originating from a change in the eluent. Not surprisingly, this practice led to fast elution of the zwitterionic X-Pro dipeptides (X = Gly, Ala, Leu, Phe and Pro) from the C-18 column. At 5 °C, no distinct dynamic phenomena were observed, although the unusually low peak efficiency (broad, tailing peaks) hinted at interconversion phenomena. It is suspected that relatively slow interconversion occurs but the separation power of the hydrophobic stationary phase under the given mobile phase conditions is not sufficient to achieve isomer separation.

Under identical mobile phase conditions, the X-Pro dipeptides showed rather short retention but distinctly interconverting elution profiles on a non-chiral zwitterionic column designed to retain strongly polar solutes in HILIC mode by a sulfobetaine modification. The isomerization rates calculated from the elution profiles at 5 °C were 3–8 times lower than on the chiral zwitterionic column (Fig. 7) and in better agreement with isomerization parameters obtained for X-Pro peptides with other methods (see Section 3.3). These findings support the hypothesis that the ZWIX-type CSP facilitates the interconversion by inducing conformational adjustments in the peptide or, although this aspect could not be unambiguously verified, by engaging the analytes or rather parts thereof in solvophobic interactions which support the formation of the uncharged (with respect to the amide nitrogen and oxygen) transition state of the isomerization. However, one has to bear in mind that the acidic and basic interaction sites of the chiral (tertiary amine) and achiral (quaternary amine) stationary phases may differ considerable in strength and accessibility, which may at least partially account for the different results.

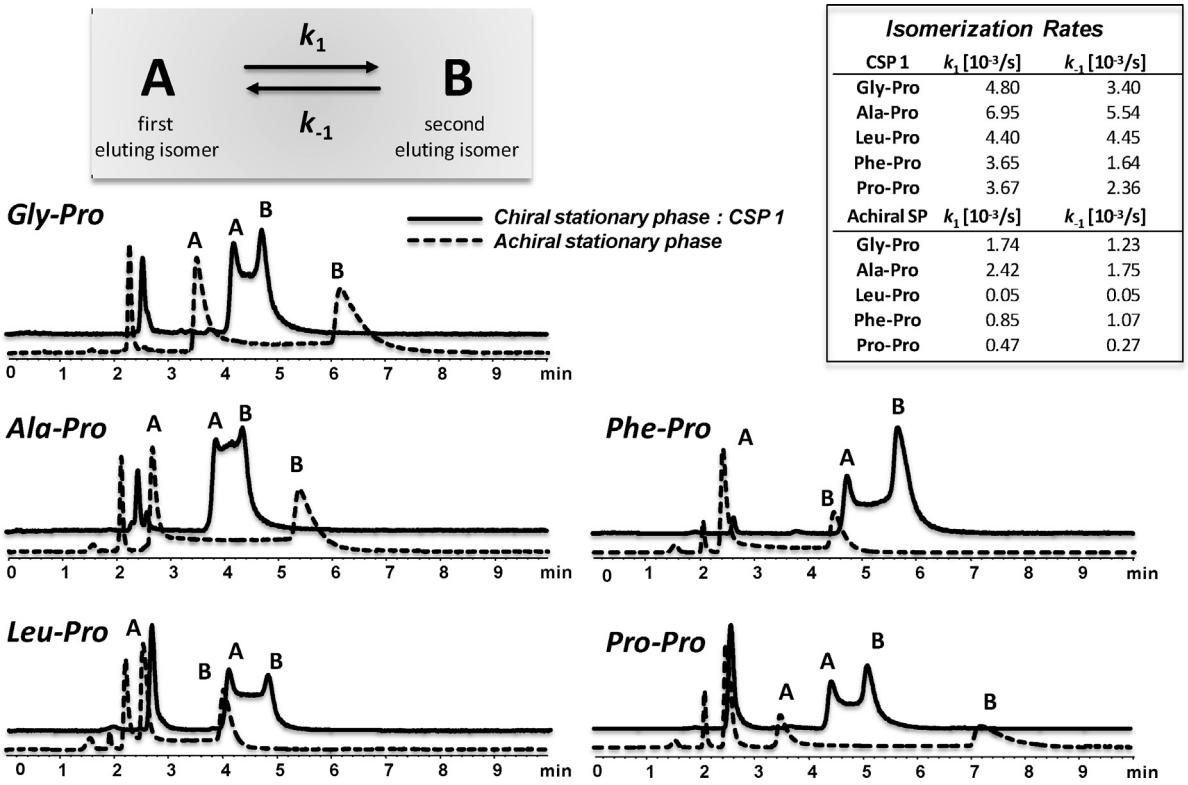
Separating chromatographic, kinetic and thermodynamic effects (especially potential catalytic influences of the selector) exceeded the scope of this study. Instead we focused on the determination of kinetic and thermodynamic parameters for the given system and their comparison with data obtained with other techniques.

The determination of the isomerization parameters k_1, k_{-1}, $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ of the individual peptides is described in detail in the following sections.

4.3. Determination of kinetic reaction rates from dynamic elution profiles

The DCXporer software [43], which is based on the stochastic model of chromatography, was developed by O. Trapp for the calculation of kinetic parameters of (pseudo-) first order reactions from chromatograms of interconverting stereoisomers. Despite potential catalytic effects from the selector, the on-column isomerization of X-Pro peptides complies with the requirement of first order reaction kinetics because the concentration of the chiral selector remains constant throughout the experiment due to its covalent attachment to the stationary phase.

In principle, the *cis–trans* isomerization of X-Pro peptides is a reversible reaction with an equilibrium constant determined by the experimental parameters outlined above. The two ground states, however, are energetically different and the kinetic rate constants of the forward and backward reaction are generally expected to differ from each other (see below).



Isomerization Rates

CSP 1	k_1 [10⁻³/s]	k_{-1} [10⁻³/s]
Gly-Pro	4.80	3.40
Ala-Pro	6.95	5.54
Leu-Pro	4.40	4.45
Phe-Pro	3.65	1.64
Pro-Pro	3.67	2.36
Achiral SP	**k_1 [10⁻³/s]**	**k_{-1} [10⁻³/s]**
Gly-Pro	1.74	1.23
Ala-Pro	2.42	1.75
Leu-Pro	0.05	0.05
Phe-Pro	0.85	1.07
Pro-Pro	0.47	0.27

Fig. 7. Comparison of isomerization rates on a chiral (CSP 1, solid lines) and achiral (dotted lines) zwitterionic stationary phase at 5 °C. The kinetic rate constants of the forward (A→B, k_1) and backward (B→A, k_{-1}) isomerization are significantly higher on CSP 1, suggesting a catalytic influence of the chiral selector. Rate constants were determined directly from the chromatograms using the DCXplorer software.

Looking at the processes from the angle of the theoretical plate model, the *cis–trans* equilibrium constant in the mobile phase (K_{AB}^{mob}) is not affected by the immobilized selector. The equilibrium constant in the stationary phase (K_{AB}^{stat}) is, however, anticipated to deviate significantly from that of the free peptide due to the adsorption–desorption processes involving both the selector and other, non-specific adsorption sites (For the sake of clarity, no further distinction into stereoselective and non-stereoselective stationary phase effects is undertaken).

As a result of the different equilibrium constants, the kinetic rate constants of interconversion in the stationary phase (k^{stat}) will deviate from those of the peptides in solution (k^{mob}) but the individual constants for the mobile and stationary phases are not directly accessible from chromatographically obtained data. The observed rate constants of the forward (k_1) and backward (k_{-1}) reaction are combined averages representing the whole chromatographic system.

Compared to a solution of the free peptides, the situation is further complicated by the superimposed chromatographic separation process: isomer separation is the result of a difference in the Gibbs energies of the complex formation of the peptide rotamers with the chiral selector ($\Delta\Delta G^{\ddagger} \neq 0$ for the complex formation). The different degrees of complex formation cause a difference in the isomerization dynamics of the two rotamers in the stationary phase: The first eluting isomer is depleted faster during the chromatographic separation ($k_1^{stat} > k_{-1}^{stat}$). On the other hand, the second eluting species is depleted to a larger extent due to its longer residence time in the stationary phase. These effects cancel each other out and the observed isomerization equilibrium is assumed to remain unperturbed by the separation. Nonetheless, elaborately designed experiments are required to deconvolute the contributions of achiral mobile and chiral stationary phase equilibria to the observed, "apparent" kinetic rate constants [53,54].

It is essential to bear in mind that the reaction rates discussed in this contribution are not universally valid but describe the isomerization of X-Pro peptides *within this particular chromatographic system* [54], that is, on the *Cinchona*-sulfonate-based zwitterionic CSP 1. Nonetheless, they are suitable for comparison with data obtained with solution-phase methods such as NMR or electrophoresis, and allow for the estimation, if not full quantification, of accelerating or decelerating effects of the separation system on the interconversion [55]. Using the specialized software, the kinetic rate constants $k_{A\to B}$ and $k_{B\to A}$ for the isomerization of X-Pro peptides on CSP 1 were determined directly from the chromatograms in the temperature range in which interconverting elution profiles were observed (Fig. 8 and Table 1). Subsequently, isomerization barriers of the forward and backward reactions, $\Delta G_{AB}^{\ddagger}$ and $\Delta G_{BA}^{\ddagger}$, were calculated from the reaction rates using the Eyring equation (Eq. (2) and Section 4.4).

For the reasons outlined above, the following discussion is largely limited to the kinetic reaction rate constant of the forward reaction (conversion of the first eluting isomer A into the second eluting isomer B).

The kinetic isomerization rates presented in Table 1 are of comparable magnitude as those generally reported for X-Pro peptides (ca. 10^{-2}–10^{-3} s^{-1}, [21]). However, they are definitely higher than those obtained using other methods such as CZE or HPLC in aqueous systems (2–10 × 10^{-5} s^{-1} at ca. 0 °C [25,30], 4–8 × 10^{-3} s^{-1} at 22 °C [56]), which can be interpreted as a supporting influence of the chromatographic system and presumably the chiral selector itself.

As expected, X-Pro dipeptides with less sterically demanding *N*-terminal amino acids, in particular Ala and Gly, isomerize faster than Phe-Pro and Pro-Pro. Leu-Pro bears a more bulky, but rather flexible *N*-terminal side chain, which gives rise to medium isomerization rates. Gly-D-Pro and Phe-Pro have been pointed out earlier (see Section 3.2 and supplementary material) for their

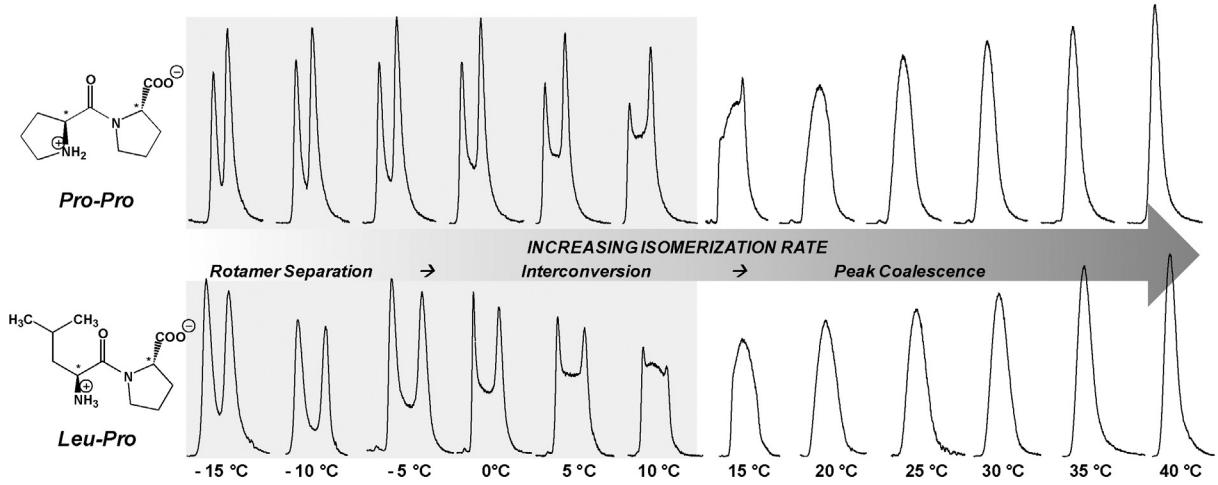


Fig. 8. Elution profiles of interconverting Phe-Pro (upper panel) and Leu-Pro (lower panel) on CSP 1. The gray areas mark the chromatograms used for obtaining the kinetic rate constants.

chromatographic peculiarities. They were noticeable again as having the highest (Gly-D-Pro) and lowest (Phe-Pro) kinetic rates of interconversion. The relatively slow isomerization of Phe-Pro has previously been observed in CZE applications [30].

It is hardly surprising that the on-column isomerization rates showed strong temperature-dependence. At temperatures at or above 0 °C, they were very high for all peptides, especially for Gly-D-Pro (1.2×10^{-2} s^{-1} at 10 °C, with $\alpha_{rot} = 1.1$). The apparent rate constant determined for this peptide is higher than literature values for Gly-Pro [in the absence of a chiral influence] and suggests that the chiral selector promotes the isomerization, possibly by facilitating the apolar transition state through solvophobic interactions.

A comparison of the rate constants obtained for the enantiomeric peptides Gly-L-Pro and Gly-D-Pro supports the concept of a directing effect of the chiral selector: Distinctly higher isomerization constants were determined for Gly-D-Pro than for its enantiomer Gly-Pro, which in the absence of a chiral field would exhibit identical isomerization behavior. Two scenarios are conceivable: In the first, Gly-Pro forms an energetically preferred, and thus stronger, complex with the chiral selector dominated by the electrostatic interaction (double ion pairing). The ability of Gly-Pro to isomerize is impaired by this attachment. The slightly higher isomerization barrier ($\Delta G^{\ddagger}$, Table 2) determined for Gly-Pro as compared to Gly-D-Pro seemingly points in that direction. Nonetheless, the retention factors of Gly-Pro are smaller than those of Gly-D-Pro, which contradicts this scenario and speaks in favor of the second, which assumes similar electrostatic interactions for the two enantiomers with the selector but a discriminating influence of weaker, attractive non-covalent interactions, possibly solvophobic/dispersive ones which favor the attachment of Gly-D-Pro to the selector and support its isomerization.

The ratio of the reaction rate constants was observed to shift as the temperature was increased. k_1:k_{-1} increased strongly for Gly-D-Pro and Phe-Pro and decreased for all other peptides. For Leu-Pro, the two rate constants were rather similar even at low temperatures. k_{-1} increased strongly with temperature and the ratio almost reached unity at 10 °C. This observation reflects the shifting peak area ratio but, as stated above, it is not appropriate to conclude from the larger relative peak area that the second eluting isomer is the *trans*-configured one. Neither should the stronger increase of the backward reaction rate with temperature be interpreted as the manifestation of increasing conversion of the thermodynamically disfavored isomer (*cis*) at increasing temperature. As illustrated by a dynamic CZE experiment performed by Schötz et al. in which over 80% of *cis*-Phe-Pro were found [25], the separation system can give rise to entirely unexpected isomerization equilibria and dynamics. In our chromatographic system, salt formation, ion pairing and attachment of the peptide to the selector might encourage the peptide to assume *cis*-conformation. Stereospecific adsorption/desorption processes could also change the isomerization dynamics compared to a peptide in solution. In this context, a chiroptical detection principle such as circular dichroism may be employed in order to facilitate correct peak assignment as opposed to the non-specific charged aerosol detector. On the other hand, the unprotected X-Pro dipeptides lack suitable chromophores and would have to be derivatized to facilitate such a method. A derivatization approach, in turn, is likely to change the isomerization dynamics compared to the free peptide and introduce an additional element of uncertainty.

Table 1
Kinetic reaction rate constants for X-Pro peptides at sub-ambient temperatures. k_1: kinetic rate constant of the forward reaction (conversion of first eluting isomer A to second eluting isomer B), k_{-1}: kinetic rate constant of the backward reaction. Chromatographic conditions: CSP 1, 150 × 3 mm i.d., mobile phase: methanol, 50 mM formic acid, 25 mM diethylamine, 0.4 mL min^{-1}, temperature range: −15 °C to +15 °C, cryostat. n.d.; not determined (peak coalescence).

T [°C]	Ala-Pro	Gly-Pro	Gly-D-Pro	Leu-Pro	Phe-Pro	Pro-Pro
Reaction rate k_1 [10^{-3} s^{-1}]						
−15	0.33	1.55	n.d.	0.59	0.12	0.71
−10	2.38	1.44	3.99	1.15	0.44	0.79
−5	3.22	1.91	5.02	2.01	1.02	1.62
0	4.87	3.16	6.28	2.90	2.11	2.24
5	6.95	4.80	6.85	4.40	3.65	3.67
10	n.d.	7.03	12.09	6.67	5.65	4.68
15	n.d.	n.d.	n.d.	n.d.	n.d.	6.66
Reaction rate k_{-1} [10^{-3} s^{-1}]						
−15	0.23	1.01	n.d.	0.50	0.05	0.42
−10	1.75	1.08	3.43	1.04	0.21	0.52
−5	2.52	1.45	4.29	1.85	0.47	1.06
0	3.80	2.43	4.87	2.83	1.43	1.48
5	5.54	3.40	4.83	4.45	1.64	2.36
10	n.d.	4.86	7.16	6.62	2.14	3.35
15	n.d.	n.d.	n.d.	n.d.	n.d.	4.73

4.4. Determination of activation parameters of the isomerization

The activation enthalpy $\Delta H^{\ddagger}$ and entropy $\Delta S^{\ddagger}$ for the isomerization of X-Pro peptides on ZWIX CSPs were obtained from the slope and y-intercept of Eyring plots ($\ln(k_1 \cdot T^{-1})$ vs. T^{-1}, Eq. (1)). The respective data for all peptides under investigation and the activation parameters $\Delta H^{\ddagger}$, $\Delta S^{\ddagger}$ and $\Delta G^{\ddagger}$ are summarized in Table 2.

Table 2
Parameters of Eyring plots and thermodynamic activation parameters for the isomerization of X-Pro peptides. A: first eluting isomer, B: second eluting isomer (conformation not assigned). Bold numbers indicate the dominating contribution to the isomerization barrier. Chromatographic conditions: CSP 1, 150 × 3 mm i.d., mobile phase: methanol, 50 mM formic acid, 25 mM diethylamine, 0.4 mL min^{-1}, temperature range: −15 °C to +15 °C, cryostat, CAD.

Peptide	Eyring plots (A→B)			$\Delta H^{\ddagger}$ [kJ mol^{-1}]	$\Delta S^{\ddagger}$ [J K^{-1} mol^{-1}]	$\Delta G^{\ddagger}$ (0 °C) [kJ mol^{-1}]	$\Delta G^{\ddagger}$ (Ref. [25]) [kJ mol^{-1}]
	Slope	Intercept	R^2				
Ala-Pro	−5043.6	7.5237	0.9951	**41.93**	−135	78.8	86.1 (*trans*→*cis*)85.3 (*cis*→*trans*)
Gly-Pro	−5824.2	9.9548	0.9921	**48.43**	−115	79.8	
Gly-D-Pro	−3474.0	2.0780	0.9040	28.88	**−180**	78.2	
Leu-Pro	−6635.2	12.825	0.9914	**55.17**	−91	80.0	86.3 (*trans*→*cis*)89.3 (*cis*→*trans*)
Phe-Pro	−9295.1	22.126	0.9872	**77.28**	−14	80.7	85.6 (*trans*→*cis*)90.7 (*cis*→*trans*)
Pro-Pro	−5632.8	8.9094	0.9804	**46.83**	−123	80.6	

Apparent isomerization barriers $\Delta G^{\ddagger}$ for the *cis–trans* isomerization of the X-Pro bond at 0 °C were determined to be around 80 kJ mol^{-1}, which is similar, although slightly lower than activation energies reported for X-Pro peptides in the literature. For example, Schoetz et al. found $\Delta G^{\ddagger}_{trans\rightarrow cis}$ of ca. 86 kJ mol^{-1} in CE experiments with Ala-Pro, Leu-Pro and Phe-Pro (see Table 2). Other methods, among them HPLC-based ones, also yielded higher activation barriers [8,25,30]. To the best of our knowledge, isomerization barriers lower than the ones reported here have not been observed before but for reasons given at the beginning of the previous section (influence of the mobile phase, selector loading, phase ratio), one must be very careful in interpreting these results as unequivocal evidence for a catalytic influence of the chiral selector.

The thermodynamic activation barriers of the individual X-Pro peptides are similar but the connection between $\Delta G^{\ddagger}$ and the bulkiness of the side chain of the *N*-terminal residue is evident. Increasing steric hindrance exerted by the *N*-terminal residue during the rotation of the amide bond from Gly- and Ala-Pro over Leu-Pro to Pro-Pro is reflected in the increasing isomerization barriers. As expected, the bulkiness of the side chain and the stabilizing effect of the aromatic residue on the *cis*-conformer of Phe-Pro resulted in comparatively high activation energy of the rotation [44].

While the isomerization barriers of the peptides investigated were rather similar, we observed considerable differences in the enthalpic ($\Delta H^{\ddagger}$) and entropic ($\Delta S^{\ddagger}$) contributions and depending on the *N*-terminal amino acids (Table 2). Generally speaking, positive values for the isomerization entropy $\Delta S^{\ddagger}$ point toward a (energetically favorable) increase of degrees of freedom (disorder) such as the breaking of hydrogen bonds from the solvation shell or conformational restraints [54].

Consequently, increasingly negative $\Delta S^{\ddagger}$ values for the on-column interconversion of Phe-Pro through Gly-D-Pro can be interpreted as originating from increasing solvation effects for the respective peptides and/or salt formation of their charged moieties (possibly with ionic additives) in the course of the isomerization process and particularly after the breaking of stabilizing electrostatic interactions involving the charged termini [19]. Significant increments to the observed isomerization entropy may also be generated by the selector–peptide interaction.

High, negative values for the activation entropy $\Delta S^{\ddagger}$ are often considered indicative of a dissociative mechanism [57], but this in not in accordance with the transition state of the *cis–trans* isomerization of X-Pro. It is more likely that particularly strong solvation effects and contributions from the peptide–selector attachment are the reason for negative isomerization entropies. This hypothesis is supported by the findings of Schötz et al., who reported positive isomerization entropies for Ala-Pro, Leu-Pro and Phe-Pro in an aqueous CZE system (i.e., in solution) [25]. The unusually high, negative $\Delta S^{\ddagger}$ value obtained for Gly-D-Pro, in particular in comparison with its enantiomer Gly-L-Pro, reveals significant contributions from the chiral surface modification.

Isomerization enthalpy $\Delta H^{\ddagger}$ shows a trend inverse to that of the entropy contributions. Large, negative entropy and small, positive enthalpy contributions result in isomerization barriers similar to those originating from small, negative entropy and large, positive enthalpy. For example, $\Delta H^{\ddagger}$ was calculated to be ca. 29 kJ mol^{-1} for Gly-D-Pro and ca. 77 kJ mol^{-1} for Phe-Pro but the resulting difference in $\Delta G^{\ddagger}$ (0 °C) was only 2.5 kJ mol^{-1} due to the respective entropic contributions (Table 2). Overall, the isomerization enthalpies represented the major contribution to $\Delta G^{\ddagger}$ (0 °C), the only exception being Gly-D-Pro. Nonetheless, $\Delta H^{\ddagger}$ values for the interconversion of X-Pro on CSP 1 were significantly lower than what literature values, again pointing toward a determining influence of the chromatographic system on the isomerization.

5. Conclusion

The data presented in this contribution demonstrate for the first time the applicability of *Cinchona*-based zwitterionic chiral stationary phases (ZWIX-type CSPs) for separations of conformational (*cis–trans*) isomers of dipeptides containing *C*-terminal proline. Chromatographic rotamer separation is feasible at temperatures below 10–15 °C depending on the peptide. Temperature increase encourages interconversion and therefore plateau formation and peak coalescence occur between 10 and 20 °C. Full coalescence was observed above ca. 20 °C, rendering higher temperatures desirable for the elimination of interconversion effects on chemo- or enantioselective separations of proline-containing peptides. The potential of ZWIX-type CSPs for preparative separations of rotamers at low temperatures remains to be further explored.

The dynamic elution profiles of interconverting rotamers were evaluated using dedicated software solutions which allow access to kinetic rate constants and thermodynamic parameters characterizing the isomerization process. The chiral selector is expected to cause a deviation of the activation parameters and possibly also the equilibrium constant of the *cis–trans* isomerization from those of the free peptide in solution. However, without accounting for phase ratio and surface coverage, it was not possible to separate stereoselective contributions from non-stereoselective influences of the buffer salt-containing mobile phase (e.g. solvation effects) and non-stereoselective adsorption on the chiral stationary phase. The results obtained with this method are therefore not universally valid parameters but describe for the first time the behavior of X-Pro peptides on a *Cinchona*-based ZWIX-type CSP. In order to fully elucidate the contribution of the selector, solution-phase experiments such as NMR and C(Z)E which investigate the isomerization of the free peptide in the presence of the chiral selector can be envisaged.

Kinetic rate constants and thermodynamic activation parameters were shown to be comparable with literature data and allowed tentative conclusions regarding the influence of the chiral stationary phase on the isomerization dynamics: According to our results, the isomerization barrier for X-Pro peptides in the chiral

system is lower than in solution. Kinetic rate constants are higher and activation entropy and enthalpy vary strongly depending on the *N*-terminal amino acid residue. The results differ considerably from results obtained with solution-phase methods. Isolation of mobile and stationary phase contributions through further systematic studies of the chromatographic system parameters (buffer salt concentration, selector coverage, endcapping groups etc.) would be required to quantify and, possibly, direct the rotamer selectivity of ZWIX-type CSPs. Further development of this method is expected to benefit greatly from molecular modeling of the peptide–selector interactions and spectroscopic (NMR) studies of the selector–selectand interactions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2013.08.004.

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