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Effects of temperature on retention of chiral compounds on a ristocetin A chiral stationary phase

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Abstract

The isocratic retention of enantiomers of chiral analytes, i.e. tryptophan, 1,2,3,4-tetrahydroisoquinoline and γ-butyrolactone analogs, was studied on a ristocetin A chiral stationary phase at different temperatures and with different mobile phase compositions, using the reversed-phase, polar-organic and normal-phase modes. By variation of the both mobile phase composition and the temperature, baseline separations could be achieved for these enantiomers. The retention factors and selectivity factors for the enantiomers of all investigated compounds decreased with increasing temperature. The natural logarithms of the retention factors (ln k) of the investigated compounds depended linearly on the inverse of temperature ($1/T$). van't Hoff plots afforded thermodynamic parameters, such as the apparent change in enthalpy (ΔH°), the apparent change in entropy (ΔS°) and the apparent change in Gibbs free energy (ΔG°) for the transfer of analyte from the mobile to the stationary phase. The thermodynamic parameters (ΔH°, ΔS° and ΔG°) were calculated in order to promote an understanding of the thermodynamic driving forces for retention in this chromatographic system.

Keywords: Chiral stationary phases, LC; Enantiomer separation; Temperature effects; Retention behaviour; Ristocetin

1. Introduction

The search for new and effective chiral selectors, capable of separating a wide variety of enantiomeric compounds, is an ongoing process. In the past few years, macrocyclic antibiotics have been shown to be an exceptionally useful class of chiral selectors for the separation of enantiomers of biological and pharmacological importance. Ristocetin A is one of the most recently introduced chiral selectors from this class [1–3]. This compound consists of an aglycon portion with four joined macrocyclic rings (one 12-membered, one 14-membered and two 16-membered rings) to which several sugars (including arabinose, glucose, mannose and rhamnose) are covalently attached. Ristocetin A, with 38 stereogenic centers, 7 aromatic rings, 6 amide linkages, 21 hydroxy groups, 2 primary amine groups, and 1 methyl ester, is the most complex of the macrocyclic glycopeptides used in chiral stationary phases (CSPs). All of the various functional groups are useful for chiral recognition through hydrophobic interactions, π–π interactions, charge–charge

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(electrostatic) interactions, hydrogen bonding, dipolar interactions and steric interactions. The ristocetin-A-containing stationary phase is a multimodal CSP; it can be used in the reversed-phase (RP) mode, the polar-organic (PO) mode and the normal-phase (NP) mode.

In all chromatographic modes, mainly the concentration and nature of the mobile phase components, together with other variables, such as the flow-rate and the pH of the mobile phase, control the selectivity and retention factors. Significant effects can also be seen by altering the temperature. Interest in the use of temperature as a separation variable has increased, particularly in RP high-performance liquid chromatography (RP-HPLC) [4–15]. The results suggest that there are at least two different effects of temperature that can affect resolution. One effect is the influence on the viscosity and on the diffusion coefficient of the solute. This is largely a kinetic effect, which improves the efficiency (i.e. peak width) [16]. The mobile phase mass transfer is improved because an increase of temperature reduces the viscosity of the mobile phase and also increases the diffusion coefficient of the solute in both the mobile and the stationary phase. Another completely different temperature effect is the change in the separation factor (α), which is related to the peak-to-peak separation distance. The separation factor usually decreases as the temperature is increased. This occurs because the partition coefficients and therefore the Gibbs free energy change (ΔG^{o}) of transfer of the analyte between the stationary phase and the mobile phase vary with temperature. This is the thermodynamic effect. The effect of temperature on selectivity is controversial and this is partly due to our lack of understanding as to how the temperature alters the enthalpy change associated with the transfer of solutes from the mobile to the stationary phase for different systems. Horvath et al. [8] and Zhu et al. [9] pointed out that the impact of the enthalpy change on selectivity is still far from clear.

Enantioselective retention mechanisms are sometimes influenced by temperature to a greater extent than are ordinary separations. This has been noted for some time in chiral gas chromatography [17–21]. In addition, it is known that these are achiral and chiral contributions to retention that can vary with a wide variety of experimental parameters [22–26].

In the past few years, temperature effects in liquid chromatography have been studied for different CSPs, such as those based on chiral crown ethers, cyclodextrins, α_1-acid-glycoprotein, π-complex molecules, derivatized cellulose and chiral macrocyclic antibiotics [27–38].

The aim of the present paper was to investigate the effects of temperature and mobile phase composition on enantioselective separations with a ristocetin-A-containing CSP, the Chirobiotic R™ column. The chromatographic separations were carried out in all three chromatographic modes, i.e. RP, PO and NP modes. The model compounds investigated were the racemic, D,L-tryptophan (Trp) (**1**), *erythro*- and *threo*-D,L-β-methyltryptophan (β-MeTrp) (**2,3**), *N*-carbobenzyloxy-D,L-tryptophan (CBZ-Trp) (**4**), *N*-(3,5-dinitro-2-pyridyl)-D,L-tryptophan (DNPyr-Trp) (**5**), *R,S*-1-[5-chloro-2-(methylamino)phenyl]-1,2,3,4-tetrahydroisoquinoline (CMP-Tic) (**6**) and *R,S*-γ-phenyl-γ-butyrolactone (**7**) (Fig. 1). These solutes exhibited a charged form or were neutral under the conditions investigated. The separation of the enantiomers of these compounds by HPLC using CSPs was reported previously [1–3].

Considerable variation of the mobile phase composition and of the temperature was utilized to determine the thermodynamic parameters. Retention data obtained at different temperatures were used for an evaluation of the variations in enthalpy (ΔH^{o}) and entropy (ΔS^{o}), which govern the free energy of transfer (ΔG^{o}) of the analyte from the mobile to the stationary phase.

2. Experimental

2.1. Apparatus

The HPLC measurements were carried out on two Waters systems. One of them consisted of an M-600 low-pressure gradient pump, an M-996 photodiode-array detection system and a MILLENNIUM[32] chromatography manager data system. The Waters Breeze system consisted of a 1525 binary pump, a 487 dual channel absorbance detector, a 717 plus autosampler and BREEZE data manager software (both systems from Waters Chromatography, Milford, MA, USA). Both chromatographic systems were equipped with Rheodyne Model 7125 injectors (Cotati, CA, USA) with 20-μl loops.

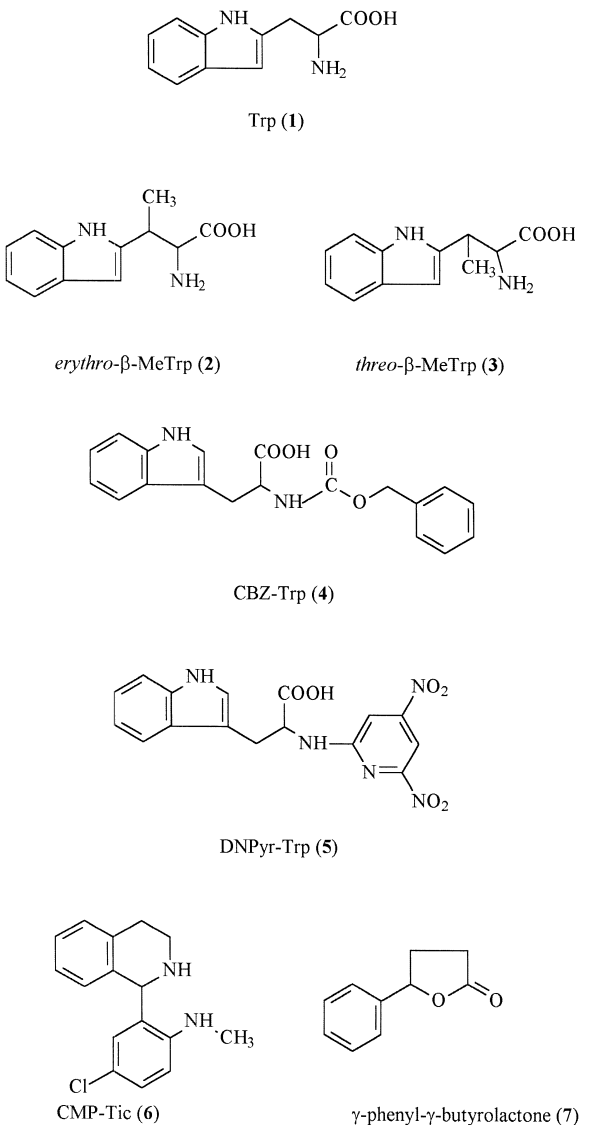

Fig. 1. Structures of investigated analytes: **1**, D,L-tryptophan (Trp), **2**, *erythro*-D,L-β-MeTrp (β-MeTrp), **3**, *threo*-D,L-β-MeTrp (β-MeTrp), **4**, *N*-carbobenzyloxy-D,L-tryptophan (CBZ-Trp), **5**, *N*-(3,5-dinitro-2-pyridyl)-D,L-tryptophan (DNPyr-Trp), **6**, *R,S*-1-[5-chloro-2-(methylamino)phenyl]-1, 2, 3, 4-tetrahydroisoquinoline (CMP-Tic), **7**, *R,S*-γ-phenyl-γ-butyrolactone.

The column used for analytical separation was a ristocetin-A-containing Chirobiotic R™ column, 250×4.6 mm I.D., 5 μm particle size (Astec, Whippany, NJ, USA). The column was thermostated in a waterbath, applying a cooling–heating thermostat (MK 70, Mechanik Prüfgeräte, Medlingen, Germany). The precision of temperature adjustment was ±0.1 °C.

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

2.2. Chemicals and reagents

Trp (**1**), CBZ-Trp (**4**) and DNPyr-Trp (**5**) were purchased from Sigma (St. Louis, MO, USA) and CMP-Tic (**6**) and *R,S*-γ-phenyl-γ-butyrolactone (**7**) were from Aldrich (Milwaukee, WI, USA) (Fig. 1). The racemic *erythro*-D,L- (**2**) and *threo*-D,L-β-MeTrp (**3**) were prepared in our laboratory [39]. For identification of the *threo*-D- and *erythro*-D-enantiomers of β-methyl amino acids, L-amino acid oxidase digestion of racemic *erythro* or *threo* compounds was applied [40].

Methanol (MeOH), acetonitrile (MeCN), ethanol (EtOH) and *n*-hexane (Hex) were of HPLC grade, while glacial acetic acid (HOAc), triethylamine (TEA) and other chemicals were of analytical reagent grade (from Merck Darmstadt, Germany). Ultrapure water was obtained from a Millipore Milli-Q system (Milford, MA, USA) and was used for the preparation of all aqueous solutions.

Triethylammonium acetate (TEAA) 0.1% buffers were prepared by titration of 0.1% (by volume) aqueous solutions of TEA with HOAc to a suitable pH. Mobile phases for RP, PO and NP chromatography were prepared by mixing the indicated volumes of buffers and/or solvents and were further purified by filtration through a 0.45-μm Millipore filter, type HV (Molsheim, France). The eluents were degassed in an ultrasonic bath, and helium gas was purged through them during the analyses.

3. Results and discussion

3.1. Effect of mobile phase composition and temperature in reversed-phase separation

The investigated Trp analogs contain ionizable groups and differ from one another in hydrophobicity and the bulkiness of their substituents. The p*K* values

for Trp are pK_1~2.3 and pK_2~9.4 [41]. All *N*-substitutions in Trp (**2–5**) had little effect on pK_a values of carboxy group [42]. *N*-Carbobenzyloxy substitution of Trp (**4**) produces a compound with an amide rather than an ionizable amine group. The p*K* for the secondary amine group of *N*-(3,5-dinitro-2-pyridyl)-substituted Trp (**5**) can be estimated by analogy to a nitro- or halogen-substituted aniline [43]. The p*K* of aniline (pK=4.42) [44] is reduced by the strong electron-withdrawing effect of the nitro groups and must be less than 4.4. Thus, at the operating pH (0.1% TEAA, pH 6.5), Trp and its analogs (**1–3**) were in zwitterionic form or were negatively charged (**4** and **5**).

Enantiomeric separations on this CSP are affected by variation of the mobile phase composition and temperature. Table 1 provides chromatographic data on the RP separation for the enantiomers of the analytes depicted in Fig. 1 in mobile phases of 0.1% TEAA–MeOH (80:20, 60:40, 40:60 and 20:80, v/v) at 293 K. Table 2 provides chromatographic data for Trp (**1**) and CBZ-Trp (**4**) at six different temperatures. Two basic types of chromatographic behavior can be discerned from these data (only a part of the data is shown in Tables 1 and 2). The first type of behavior is shown in Fig. 2A, and is observed for Trp (**1**) and *erythro*- and *threo*-β-MeTrp (**2,3**). When starting from 5% (v/v) MeOH concentration, and increasing the MeOH content in the mobile phase, *k* first decreases, and then at higher MeOH content, an increase in *k* was observed. At all temperatures, such U-shaped curves were obtained. The decrease in *k* with increasing MeOH concentration is the characteristic behavior when hydrophobic interaction between the analyte and the CSP are dominant. The increase in *k* at higher MeOH content can be attributed to the lower solubility of the amino acid in MeOH. The analyte therefore favored the stationary phase, which resulted in higher retention. The selectivity (α) exhibited a slight variation with change of the mobile phase composition and temperature and for analytes **1–3** was in the range α=1.06–2.06 and no definite trend was observed with increasing MeOH concentration. The resolution for Trp was highest at 40 and 60% (v/v) MeOH content (Table 2), while for *erythro*-β-MeTrp no definite trend was observed. For *threo*-β-MeTrp, the resolution exhibited a shallow U-shaped curve, with a minimum at a mobile phase composition near 0.1% TEAA–MeOH (40:60, v/v). In this region, the two *threo*-enantiomers were not separated.

Table 1
Retention factors (k), separation factors (α) and resolutions (R_S) of enantiomers of analytes **1–7** at 293 K in reversed-phase mode at different mobile phase compositions

Chromatographic data	Mobile phase composition, 0.1% TEAA (pH 6.5)–MeOH (v/v)			
	80:20	60:40	40:60	20:80
Trp				
k_L	3.06	2.48	2.56	4.33
α	1.43	1.60	1.66	1.36
R_S	1.40	2.87	2.62	2.00
erythro-β-MeTrp				
k_L	2.29	1.67	1.74	2.18
α	1.57	1.60	1.62	1.60
R_S	1.28	2.87	0.93	1.69
threo-β-MeTrp				
k_L	2.97	2.58	2.65	3.00
α	1.68	1.29	1.00	1.13
R_S	2.11	1.31	0.00	0.50
CBZ-Trp				
k_1	7.38	1.67	0.65	0.60
α	4.31	5.79	4.80	3.45
R_S	5.12	5.95	5.22	3.57
DNPyr-Trp				
k_1	–	4.26	1.58	0.88
α	–	11.50	8.52	5.11
R_S	–	11.36	7.89	6.10
CMP-Tic				
k_1	–	5.58	2.83	1.40
α	–	2.99	2.66	3.20
R_S	–	5.90	6.76	10.17
γ-Phenyl-γ-butyrolactone				
k_1	2.94	1.36	0.95	–
α	1.52	1.41	1.32	–
R_S	1.67	2.52	1.65	–

Column, Chirobiotic R™; flow-rate, 1.0 ml min^{-1}; detection wavelength, 205 nm; mobile phases, aqueous 0.1% TEAA (pH 6.5)–MeOH (80:20, v/v) and 0.1% TEAA (pH 6.5)–MeOH (60:40, v/v), 0.1% TEAA (pH 6.5)–MeOH (40:60, v/v) and 0.1% TEAA (pH 6.5)–MeOH (20:80, v/v).

The *k*, α and R_S values of analytes **1–3** decreased with increasing temperature. The differences between the values measured at the lowest and highest temperatures were moderate. For Trp (**1**) and β-MeTrp enantiomers (**2** and **3**) a 2–6-fold decrease in

Table 2
Retention factors (k), separation factors (α) and resolutions (R_S) of enantiomers of Trp (**1**) and CBZ-Trp (**4**) as a function of temperature in reversed-phase mode at different mobile phase compositions

	Temperature (K)					
	276	283	293	303	313	323
Trp						
0.1% TEAA (pH 6.5)–MeOH (80:20, v/v)						
k_L	5.50	4.50	3.06	2.07	1.72	1.36
α	1.69	1.54	1.43	1.39	1.32	1.17
R_S	2.11	2.22	1.40	1.11	0.72	0.56
0.1% TEAA (pH 6.5)–MeOH (60:40, v/v)						
k_L	4.13	3.48	2.48	1.87	1.47	1.13
α	2.03	1.74	1.60	1.46	1.25	1.17
R_S	3.57	3.33	2.87	2.28	1.23	0.76
0.1% TEAA (pH 6.5)–MeOH (40:60, v/v)						
k_L	3.70	3.32	2.56	2.15	1.78	1.56
α	2.03	1.71	1.66	1.47	1.35	1.22
R_S	2.92	2.70	2.62	2.00	1.37	1.20
0.1% TEAA (pH 6.5)–MeOH (20:80, v/v)						
k_L	5.64	5.00	4.33	3.23	2.69	2.28
α	1.68	1.48	1.36	1.36	1.21	1.12
R_S	2.31	2.21	2.00	1.57	1.26	1.12
CBZ-Trp						
0.1% TEAA (pH 6.5)–MeOH (80:20, v/v)						
k_1	–	15.36	7.38	4.05	2.13	1.28
α	–	5.17	4.31	3.88	3.20	2.75
R_S	–	7.33	5.12	4.83	4.74	4.12
0.1% TEAA (pH 6.5)–MeOH (60:40, v/v)						
k_1	–	3.56	1.67	1.00	0.50	0.33
α	–	7.60	5.79	4.52	3.84	2.82
R_S	–	6.54	5.95	5.88	5.41	2.77
0.1% TEAA (pH 6.5)–MeOH (40:60, v/v)						
k_1	1.12	1.15	0.65	0.40	0.34	0.27
α	8.55	5.87	4.80	4.42	3.20	2.14
R_S	5.41	5.28	5.22	4.37	3.26	2.40
0.1% TEAA (pH 6.5)–MeOH (20:80, v/v)						
k_1	0.89	0.76	0.60	0.50	0.46	0.35
α	5.80	4.59	3.45	2.56	1.78	1.54
R_S	2.70	3.05	3.57	2.50	2.14	1.43

Column, Chirobiotic R™; flow-rate, 1.0 ml min^{-1}; detection wavelength, 205 nm; mobile phases, aqueous 0.1% TEAA (pH 6.5)–MeOH (80:20, v/v) and 0.1% TEAA (pH 6.5)–MeOH (60:40, v/v), 0.1% TEAA (pH 6.5)–MeOH (40:60, v/v) and 0.1% TEAA (pH 6.5)–MeOH (20:80, v/v).

k, α and R_S was observed with a temperature increase from 276 to 323 K.

A second, more traditional type of RP retention behavior was observed for CBZ-Trp (**4**), DNPyr-Trp (**5**), CMP-Tic (**6**) and γ-phenyl-γ-butyrolactone (**7**) as a function of MeOH content (Table 1, Fig. 2B). CBZ-Trp (**4**) and DNPyr-Trp (**5**) exhibited very high k values, especially for the second-eluted isomer in water-rich eluent and at low temperatures. The k values decreased dramatically especially for the second-eluted isomer with increasing MeOH content and with increasing temperature, owing to their higher lipophilicities and higher solubilities in a MeOH-rich mobile phase. The selectivity was much greater than for other Trp analogs and exhibited a decrease with increasing MeOH content and temperature. The greatest α values obtained for Trp and β-MeTrp enantiomers were <2, while for CBZ-Trp (**4**) and DNPyr-Trp (**5**) α reached 10–15 at low temperatures. The resolution changed in the same manner as the α value and was much higher than that obtained for Trp and β-MeTrp stereoisomers.

CMP-Tic (**6**) has two amine functions. On the base of the works of Chatterjee et al. [45] and Sipos et al. [43] the pK for the amine group in the tetrahydroquinoline must be p$K>10.5$. The pK of the other secondary amine group in the [5-chloro-2-(methylamino)phenyl] moiety of the molecule can be estimated on analogy with aniline. The pK value corresponding to the [5-chloro-2-(methylamino)phenyl] group should be more acidic than the amino group in aniline [44,46] due to the electron-withdrawing effect of the chlorine substituent. Considering these pK values, molecule **5** in this study should have a positive charge or should be neutral. To prove this assumption the chromatographic data were measured as a function of pH using a 0.1% TEAA–MeOH (20:80, v/v) mobile phase composition in the pH range pH 2.5–7.0. The results exhibited that k, α and R_S values increased with increasing pH and reached maximal values above pH 5. The inflexion point was around pH 3.5. In an electrophoretic system in phosphate buffer at pH 6.5, CMP-Tic moved toward the cathode at a rate 2.5-fold higher than the electroosmotic flow. This means that at the working pH CMP-Tic was positively charged, and from a comparison with the measurement of the pH effect, probably had a charge of $+1$.

The k values for CMP-Tic were higher than those obtained for Trp and β-MeTrp enantiomers (except at the highest MeOH content) (Table 1). The analyte

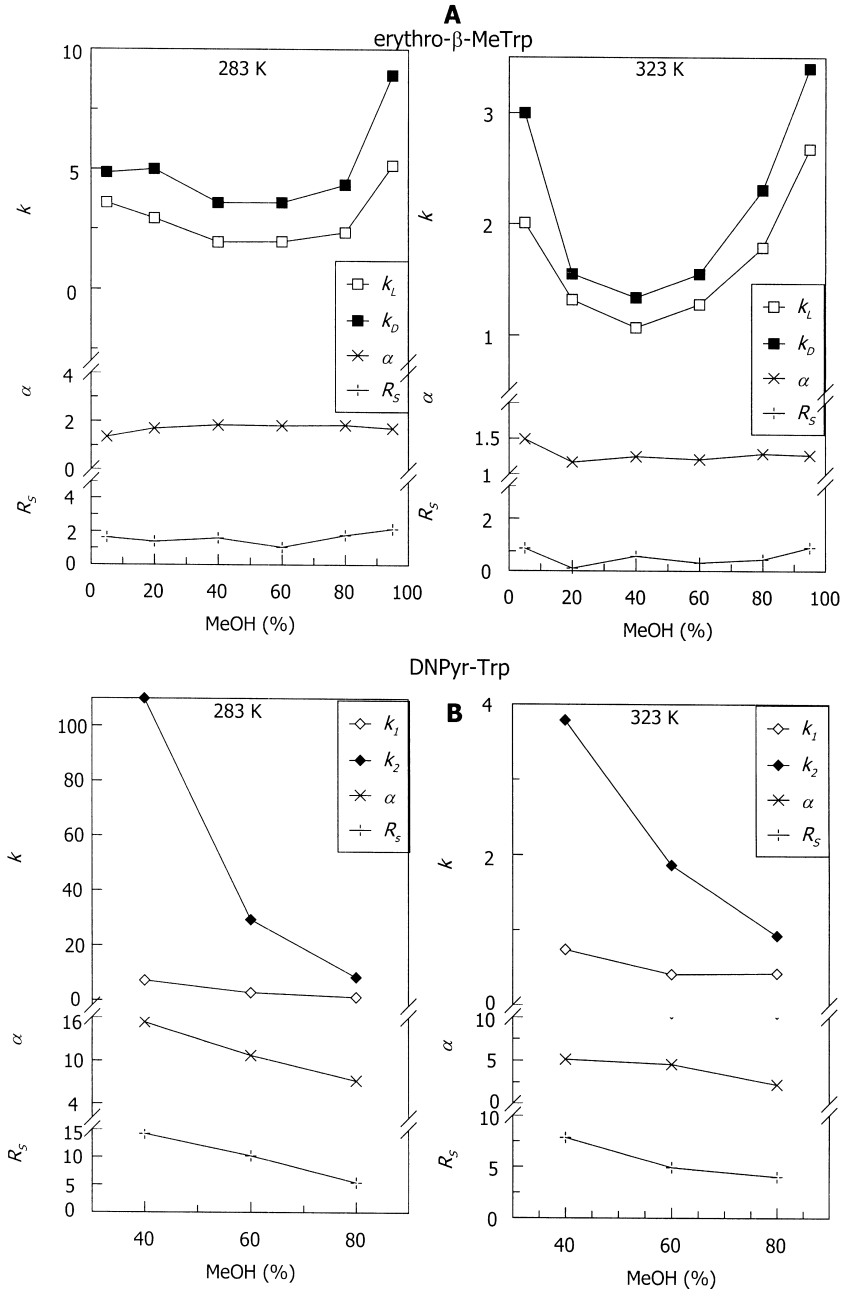

Fig. 2. Retention factors (k), separation factors (α) and resolutions (R_S) of enantiomers of *erythro*-β-MeTrp (**2**) and DNPyr-Trp (**5**) as a function of mobile phase composition and temperature. Chromatographic conditions: column, Chirobiotic R; flow-rate, 1 ml min^{-1}; detection, 205 nm; temperature, 283 and 323 K; mobile phase, aqueous 0.1% TEAA (pH 6.5)–MeOH (95:5, 80:20, 60:40, 40:60, 20:80 and 5:95, v/v); k, open symbols, first-eluted enantiomer; filled symbols, second-eluted enantiomer; □,■, *erythro*-β-MeTrp; ◇,♦, DNPyr-Trp; α, ×; R_S, +.

exhibited high retention factors despite the lack of a negatively charged group, especially in a water-rich mobile phase. This means that the dominating step in the separation of the enantiomers of CMP-Tic was not a charge–charge interaction between the stationary phase and the analyte (see below). The separation factor, α exhibited an insignificant change with increasing MeOH content, while the resolution generally increased with increasing MeOH.

In the case of CMP-Tic (**6**) the k and α values decreased with increasing temperature, while R_S exhibited an increase at higher temperatures in a water-rich mobile phase. This suggests that at higher temperature the mass-transfer and adsorption–desorption kinetics were appreciably enhanced.

γ-Phenyl-γ-butyrolactone (**7**) has no ionizable group; it is neutral at pH 6.5 and exhibited the lowest k values as of the solutes investigated (Tables 1 and 2). The α values decreased slightly with increasing MeOH concentration and temperature. The change in R_S as a function of organic modifier content and temperature was not significant as the difference between the smallest and largest R_S values did not exceed 50%.

3.2. Effect of temperature in polar-organic mode separations

The separation of enantiomers in the PO mode was carried out with a MeOH–HOAc–TEA (1000:1:1, v/v/v) mobile phase at different temperatures; the results are presented in Table 3. This mobile phase effectively separated the enantiomers of all the analytes investigated except γ-phenyl-γ-butyrolactone (**7**). The enantiomers of Trp (**1**) and β-MeTrp enantiomers (**2,3**) were separated within a short time at all temperatures. Interestingly, none of the k values changed by more than 30% between the highest and lowest temperatures. There were smaller decreases in k with increasing temperature as compared to that observed in the RP mode. Also, the selectivity (α) changed only slightly with temperature (Table 3). R_S exhibited totally different behavior from that in the RP mode; it increased with increasing temperature (Table 3).

The enantiomers of CBZ-Trp (**4**) and DNPyr-Trp (**5**) were easily separated in the PO mode, with a better selectivity at lower temperatures. Since the retention factor of the second-eluting enantiomer was not as great as that observed in the RP mode at low temperatures, the effect of temperature on the separation was modest. The R_S values for both analytes exhibited maxima as a function of temperature (observed at around 293–303 K).

The enantiomers of CMP-Tic (**6**) had very small k values, but they were separated with very high selectivity ($\alpha > 10$) at all temperatures investigated. Because of the fast elution and high selectivity, R_S decreased from a high level ($R_S = 7.28$) to a moderate value ($R_S = 1.70$) on increase of the temperature (Table 3).

3.3. Effect of temperature in normal-phase separations

The NP separation of enantiomers of analytes **2–5** was accomplished with a mobile phase of Hex–EtOH (20:80, v/v). The results are presented in Table 3. The enantiomers of Trp (**1**) and CMP-Tic (**6**) could not be separated in the NP mode, while for γ-phenyl-γ-butyrolactone (**7**) a Hex-rich eluent was needed. The results confirmed the expectation that the NP mode is closely related to the PO mode. For β-MeTrp analogs (**2,3**), the temperature had a small effect on the separation. The k and α values obtained at the lowest and highest temperatures differed to only a small extent; they decreased slightly with increasing temperature. R_S exhibited the same behavior as that observed in PO mode; it increased with increasing temperature.

The enantioseparation of CBZ-Trp (**4**) and DNPyr-Trp (**5**) in the NP mode was similar to that in the PO mode. At low temperature, they were strongly retained, but the differences in the k and α values obtained at the lowest and highest temperatures were much smaller than those in the RP mode. Despite the good α values, the R_S values for both analytes were small, and exhibited very little variation with temperature.

The enantiomers of γ-phenyl-γ-butyrolactone were not separable in Hex–EtOH (20:80, v/v). An increase in the *n*-hexane content of the mobile phase increased the retention, with parallel increases in α and R_S. In a mobile phase of Hex–EtOH (95:5, v/v), the enantiomers were partially separated. The temperature had only a small effect on both retention

Table 3
Retention factors (k), separation factors (α) and resolutions (R_s) of enantiomers of analytes **1–7** as a function of temperature in polar-organic mode at MeOH–HOAc–TEA (1000:1:1, v/v/v) mobile phase composition and in normal-phase mode at Hex–EtOH (20:80, v/v) mobile phase composition

	Temperature (K)					
	278	283	293	303	313	323
Polar-organic mode: MeOH–HOAc–TEA (1000:1:1, v/v/v)						
Trp						
k_L	1.97	1.97	1.80	1.72	1.58	1.41
α	1.76	1.60	1.65	1.55	1.50	1.44
R_S	2.08	2.18	2.60	2.66	2.55	2.48
erythro-β-MeTrp						
k_L	0.88	0.85	0.87	0.86	0.83	0.81
α	2.28	2.31	2.18	2.12	2.03	1.90
R_S	2.85	2.93	3.39	3.63	3.41	3.45
threo-β-MeTrp						
k_L	1.29	1.30	1.26	1.24	1.17	1.11
α	1.55	1.46	1.46	1.48	1.48	1.47
R_S	1.28	1.36	1.70	1.96	2.03	2.14
CBZ-Trp						
k_L	0.55	0.47	0.38	0.32	0.25	0.20
α	5.90	5.57	4.58	3.84	3.24	2.85
R_S	2.68	3.17	3.39	4.00	3.82	3.61
DNPyr-Trp						
k_1	0.72	0.65	0.52	0.42	0.35	0.29
α	4.20	3.78	3.78	3.24	3.08	2.76
R_S	4.17	4.71	4.83	4.16	4.61	3.87
CMP-Tic						
k_1	0.05	0.05	0.04	0.03	0.02	0.02
α	16.70	15.10	14.50	14.66	12.71	10.56
R_S	7.28	3.17	3.62	3.15	2.42	1.70
Normal-phase mode: Hex–EtOH (20:80, v/v)						
erythro-β-MeTrp						
k_L	0.77	0.75	0.69	0.65	0.60	0.55
α	1.95	1.90	1.84	1.77	1.73	1.69
R_S	1.55	1.83	1.76	1.82	2.58	2.35
threo-β-MeTrp						
k_L	0.76	0.75	0.68	0.64	0.60	0.56
α	1.96	1.88	1.85	1.79	1.76	1.73
R_S	1.80	1.70	2.00	2.15	2.20	2.29
CBZ-Trp						
k_L	1.79	1.77	1.43	1.27	1.07	0.95
α	2.15	1.94	1.68	1.41	1.23	1.15
R_S	0.71	0.96	0.91	0.77	0.56	0.60
DNPyr-Trp						
k_1	3.59	3.42	2.64	2.12	1.88	1.63
α	2.02	1.98	1.78	1.74	1.56	1.40
R_S	1.07	1.08	1.08	1.06	1.07	1.43
γ-Phenyl-γ-butyrolactone[a]						
k_1	11.00	9.93	8.17	6.82	5.75	4.94
α	1.07	1.08	1.06	1.04	1.04	1.02
R_S	0.58	0.61	0.65	0.62	0.58	0.46

Column, Chirobiotic R™; flow-rate, 1.0 ml min^{-1}; detection wavelength, 205 nm; mobile phase, polar-organic mode, MeOH–HOAc–TEA (1000:1:1, v:v/v), normal-phase mode, Hex–EtOH (20:80, v/v).

[a] For γ-phenyl-γ-butyrolactone, Hex–EtOH (95:5, v/v).

and selectivity. The R_S factors were also small and practically invariant with temperature.

3.4. Thermodynamic parameters for enantiomeric resolution

In order to calculate the thermodynamic parameters and to acquire information of value for an understanding of enantiomeric retention, selectivity and the mechanism on this CSP, van't Hoff plots were constructed using:

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

This expression shows that a plot of ln k versus $1/T$ has a slope of $-\Delta H^{\circ}/R$ and an intercept of $\Delta S^{\circ}/R + \ln \phi$ if ΔH° is invariant with temperature (i.e. a linear van't Hoff plot is obtained). This provides a convenient way of calculating the thermodynamic constants ΔH° and ΔS° for a chromatographic system if the phase ratio is known or can be calculated.

The corresponding $\Delta(\Delta H^{\circ})$ and $\Delta(\Delta S^{\circ})$ values can be obtained as the differences $\Delta H^{\circ}{}_2 - \Delta H^{\circ}{}_1$ and $\Delta S^{\circ}{}_2 - \Delta S^{\circ}{}_1$, or can be estimated from the selectivity factor (α), which is related to the difference in Gibbs free energy of association $\Delta(\Delta G^{\circ})$ for an enantiomeric pair, (if the temperature range investigated is narrow [26]:

$$\Delta(\Delta G^{\circ}) = -RT \ln \alpha = \Delta(\Delta H^{\circ}) - T\,\Delta(\Delta S^{\circ}) \quad (2)$$

If $\Delta(\Delta H^{\circ})$ is constant within the given temperature range, a straight line should be obtained when the natural logarithms of the α values of a given enantiomeric pair at different temperatures ($R \ln \alpha$) or $-\Delta(\Delta G^{\circ})/T$ are plotted versus $1/T$. The slope is $-\Delta(\Delta H^{\circ})$ and the intercept is $\Delta(\Delta S^{\circ})$. The $\Delta(\Delta H^{\circ})$ and $\Delta(\Delta S^{\circ})$ values obtained by the two different methods should be identical.

In the present study, all the plots of ln k vs. $1/T$ could be fitted by straight lines. Fig. 3 shows the plot of ln k vs. $1/T$ for *erythro*-β-MeTrp at a 0.1%TEAA–MeOH mobile phase composition of 60:40 (v/v). For the other analytes in all three chromatographic modes, similar straight lines were obtained with good correlation coefficients (Tables 4 and 5).

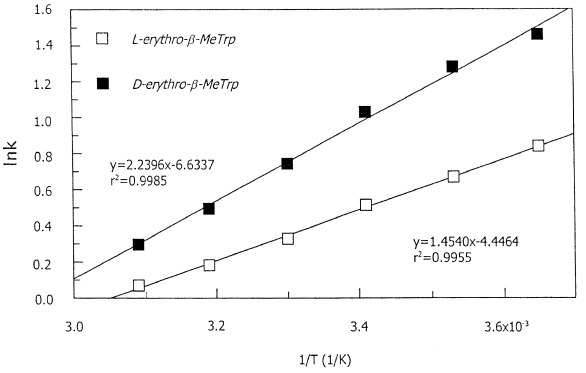

Fig. 3. Plots of natural logarithms of retention factors (ln k) of *erythro*-β-MeTrp (**2**) enantiomers as a function of the inverse of temperature ($1/T$). Column, Chirobiotic R; detection, 200 nm; flow-rate, 1 ml min^{-1}; mobile phase, 0.1% TEAA–MeOH (60:40, v/v); ln k, open symbols, first-eluted enantiomer; filled symbols, second-eluted enantiomer; □,■, *erythro*-β-MeTrp.

3.5. Thermodynamic parameters in the reversed-phase mode

Multiple, simultaneous differential interactions between the chiral analyte and a CSP are necessary in order for chiral recognition and enantioseparation to take place. Among the more important molecular interactions for chiral recognition are: hydrophobic, electrostatic, hydrogen bonding, π–π, dipolar and steric repulsion types. Ristocetin A has a plethora of functional groups, allowing it to take advantage of most of these interactions under certain conditions. For example, all of these interactions are possible in the RP mode, with the exception of the π–π interactions (which are most pronounced in nonpolar solvents). The ΔH° and ΔS° values calculated from the slopes of the plots at four different mobile phase compositions, 0.1% TEAA–MeOH (80:20, 60:40, 40:60 and 20:80, v/v), are presented in Fig. 4. The calculation of ΔS° from the intercept requires a knowledge of the phase ratio (ϕ). The determination of ϕ is relatively easy in pure liquid–liquid chromatography, but the situation is much more complex in RP chromatography with chemically bonded materials. In any case, the choice of ϕ must be in agreement with the definition of K (equilibrium constant). Different equations have been proposed for the evaluation of ϕ [5] These equations make use of technical data relating to the stationary phase

Table 4
Thermodynamic parameters $\Delta(\Delta H^{o})$ (kJ mol^{-1}) and $\Delta(\Delta S^{o})$ (J mol^{-1} K^{-1}), and correlation coefficients (r^2) of enantiomers of analytes **1–7** as a function of mobile phase composition in reversed-phase mode

	0.1% TEAA (pH 6.5)–MeOH (v/v)			
	80:20	60:40	40:60	20:80
Trp				
$\Delta(\Delta H^{o})$	−6.1	−8.5	−7.4	−5.8
$\Delta(\Delta S^{o})$	−17.1	−25.2	−21.1	−16.8
r^2_L	0.979	0.999	0.996	0.993
r^2_D	0.961	0.999	0.999	0.996
erythro-β-MeTrp				
$\Delta(\Delta H^{o})$	−7.4	−6.5	−8.0	−7.4
$\Delta(\Delta S^{o})$	−21.4	−18.2	−23.3	−21.1
r^2_L	0.998	0.996	0.999	0.994
r^2_D	0.996	0.998	0.999	0.991
threo-β-MeTrp				
$\Delta(\Delta H^{o})$	−6.4	−3.6	0.0	−1.3
$\Delta(\Delta S^{o})$	−18.0	−10.0	0.0	−3.5
r^2_L	0.998	0.997	0.999	0.991
r^2_D	0.999	0.997	0.999	0.992
CBZ-Trp				
$\Delta(\Delta H^{o})$	−11.7	−18.1	−19.3	−21.4
$\Delta(\Delta S^{o})$	−27.6	−47.2	−52.4	−62.8
r^2_L	0.999	0.996	0.959	0.988
r^2_D	0.999	0.999	0.998	0.999
DNPyr-Trp				
$\Delta(\Delta H^{o})$	–	−20.8	−16.7	−24.2
$\Delta(\Delta S^{o})$	–	−50.5	−39.4	−68.7
r^2_1	–	0.999	0.996	0.984
r^2_2	–	0.993	0.999	0.999
CMP-Tic				
$\Delta(\Delta H^{o})$	–	−5.1	−7.3	−14.4
$\Delta(\Delta S^{o})$	–	−7.9	−16.7	−39.6
r^2_1	–	0.999	0.998	0.983
r^2_2	–	0.989	0.997	0.991
γ-Phenyl-γ-butyrolactone				
$\Delta(\Delta H^{o})$	−3.6	−2.6	−1.9	–
$\Delta(\Delta S^{o})$	−8.8	−5.9	−4.0	–
r^2_1	0.999	0.996	0.993	–
r^2_2	0.999	0.998	0.993	–

Column, Chirobiotic R™; mobile phase, aqueous 0.1% TEAA–MeOH (v/v); flow-rate, 1.0 ml min^{-1}; detection wavelength, 205 nm; r^2, correlation coefficient of van't Hoff plot, ln $k - 1/T$ curves.

packing materials, such as the percentage carbon load, the bonded packing mass, the bonded alkyl chain density and the number of carbons in the alkyl ligand [5]. Any uncertainty in the phase ratio affects the ΔS^{o} values equally, and the trends in ΔS^{o} as a function of molecular structure are therefore unaffected.

Since the technical data on the Chirobiotic R column were available, ϕ could be calculated by method of Dorsey et al. [5]. When the dead volume of the column and the technical data were taken into account, ϕ was calculated at four different mobile phase compositions, i.e. at 0.1% TEAA–MeOH (80:20, 60:40, 40:60 and 20:80, v/v), and was found

Table 5
Thermodynamic parameters ΔH^{o}, ΔS^{o}, ΔG^{o}, $\Delta(\Delta H^{o})$, $\Delta(\Delta S^{o})$ and $\Delta(\Delta G^{o})$ and correlation coefficients (r^2) of analytes in polar-organic an normal-phase mode

Stereoisomer	ΔH^{o} (kJ mol^{-1})	ΔS^{o} (J mol^{-1} K^{-1})	Correlation coefficient, r^2	$\Delta G^{o}_{323\ K}$ (kJ mol^{-1})	$\Delta(\Delta H^{o})$ (kJ mol^{-1})	$\Delta(\Delta S^{o})$ (J mol^{-1} K^{-1})	$\Delta(\Delta G^{o})_{323\ K}$ (kJ mol^{-1})
Polar-organic mode							
Trp							
L	−5.5	−2.4	0.961	−4.7	−2.7	−5.1	−1.0
D	−8.1	−7.5	0.976	−5.7			
erythro-β-MeTrp							
L	−1.1	6.4	0.988	−3.2	−3.0	−3.7	−1.8
D	−4.1	2.7	0.960	−5.0			
threo-β-MeTrp							
L	−2.5	4.8	0.920	−4.0	−0.6	1.2	−1.0
D	−3.1	6.1	0.919	−5.0			
CBZ-Trp							
L	−16.3	−52.1	0.998	0.5	−12.5	−30.0	−2.8
D	−28.8	−82.1	0.999	−2.3			
DNPyr-Trp							
(1)	−15.2	−45.7	0.999	−0.4	−6.7	−12.1	−2.8
(2)	−21.8	−57.8	0.995	−3.1			
CMP-Tic							
(1)	−12.9	−59.1	0.995	6.2	−6.7	−0.7	−6.4
(2)	−19.5	−59.9	0.998	−0.2			
Normal-phase mode							
erythro-β-MeTrp							
L	−5.6	−11.9	0.995	−1.8	−2.4	−3.0	−1.4
D	−8.0	−14.9	0.999	−3.2			
threo-β-MeTrp							
L	−5.4	−11.3	0.997	−1.8	−1.6	−0.6	−1.5
D	−7.1	−11.9	0.997	−3.3			
CBZ-Trp							
L	−11.2	−28.1	0.992	−3.1	−9.7	−29.0	−0.3
D	−21.9	−57.1	0.996	−3.5			
DNPyr-Trp							
(1)	−13.9	−28.9	0.988	−4.6	−7.2	−19.4	−0.9
(2)	−21.1	−48.3	0.991	−5.5			
γ-Phenyl-γ-butyrolactone							
(1)	−13.2	−17.2	0.997	−7.6	−1.2	−3.6	−0.1
(2)	−14.4	−20.8	0.999	−7.7			

Column, Chirobiotic R™; flow-rate, 1.0 ml min^{-1}; detection wavelength, 205 nm; mobile phase, in polar-organic mode, MeOH–TEA–HOAc (1000:1:1, v/v/v); ϕ was calculated as 0.253; mobile phase in normal-phase mode, Hex–EtOH (20:80, v/v), for γ-phenyl-γ-butyrolactone Hex–EtOH (95:5, v/v); ϕ was calculated as 0.291; r^2, correlation coefficient of van't Hoff plot, ln k–$1/T$ curves.

to be 0.378, 0.405, 0.462 and 0.519, respectively. The ΔS^{o} values calculated from the intercepts of the plots via Eq. (1) are presented in Fig. 4.

The ΔH^{o} values calculated from the slopes of the plots of Eq. (1) were negative for all enantiomers. This indicates that the transfer of the enantiomers from the mobile to the stationary phase is enthalpically favored (Fig. 4). The ΔH^{o} values are in the range −5.1 to −65.3 kJ/mol. For all the investigated compounds, the enthalpy change for the sec-

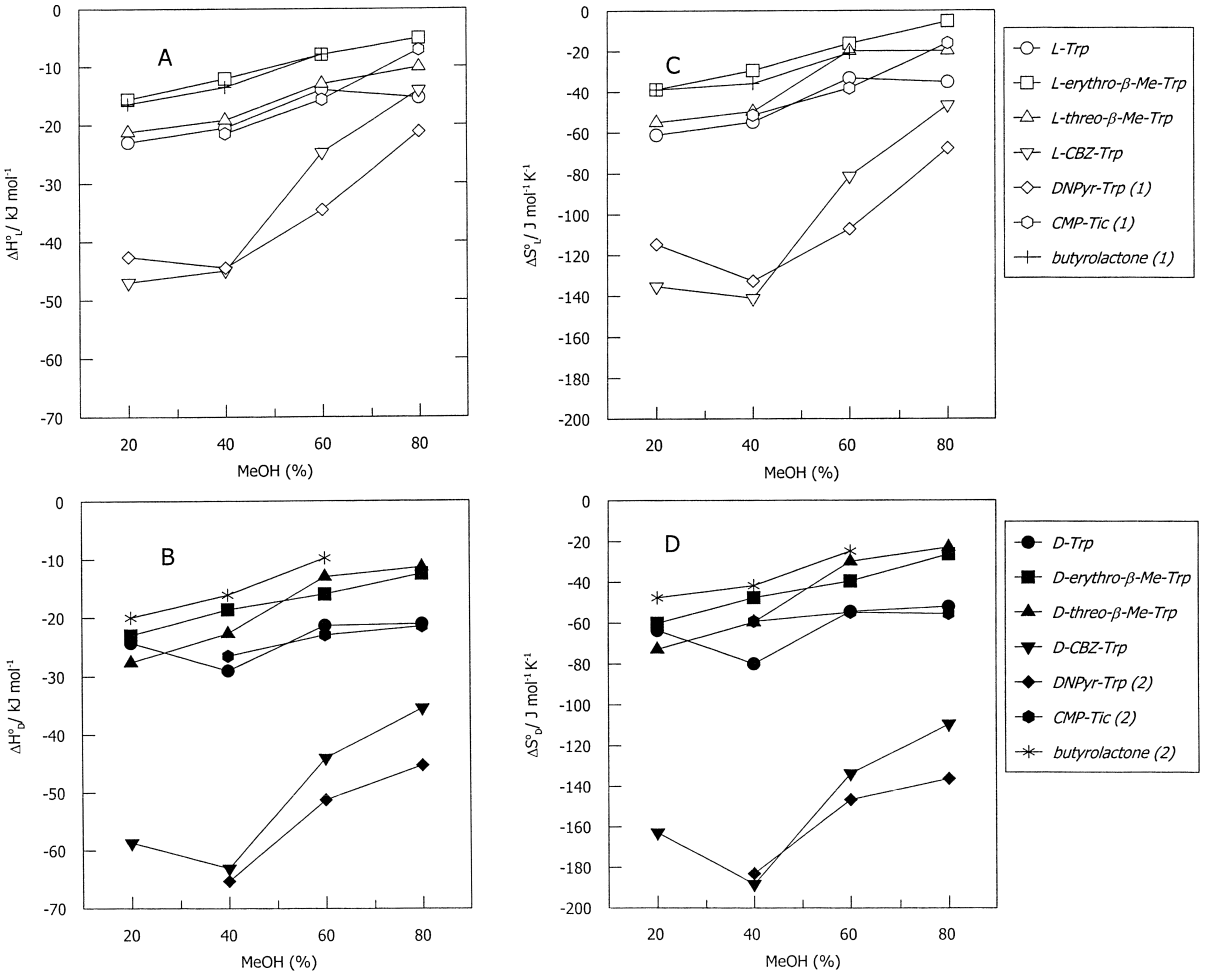

Fig. 4. Thermodynamic parameters ΔH° and ΔS° of first and second-eluted enantiomers as a function of mobile phase composition. Chromatographic conditions: column, Chirobiotic R; flow-rate, 1 ml min^{-1}; detection, 205 nm; temperature, 283–323 K; mobile phase, aqueous 0.1% TEAA (pH 6.5)–MeOH (80:20, 60:40, 40:60, 20:80, v/v); open symbols, first-eluted enantiomer; filled symbols, second-eluted enantiomer; ○, ● Trp; □, ■ *erythro*-β-MeTrp; △, ▲ *threo*-β-MeTrp; ▽, ▼ CBZ-Trp; ◇, ♦ DNPyr-Trp; ⬡, ⬢, CMP-Tic; +, ✳ γ-phenyl-γ-butyrolactone.

ond-eluted isomer is always greater (i.e. more negative) than that for the first-eluted isomer. This means that the association between the second-eluted enantiomer and the CSP is more favourable than for the first-eluted enantiomer. The second-eluted enantiomer for compounds **1–4** had the D configuration, as shown in earlier investigations [1,3]. The exact elution sequence for analytes **5,6** and **7** was not determined.

The data in Fig. 4 indicate that the entropy values (ΔS°) were negative in all cases. Also, the ΔS° values for the first-eluted enantiomer were always more positive than those for the second-eluted enantiomer (for any pair). In every case, the second-eluted enantiomers always had more negative enthalpies and, at the same time, more negative entropy values.

The two enantiomers (of a pair) must be solvated identically in the mobile phase, but may release a different number of solvent molecules when they associate with the CSP. Therefore, this contribution to ΔS° may not be identical for both enantiomers.

Since the second-eluted enantiomers have more negative ΔS^{o} values, they may have fewer degrees of freedom on the CSP (i.e. they are held at more points or are less able to move or rotate or a smaller number of solvent molecules may be displaced by the analyte when it associates with the CSP). Chiral recognition requires three or more points of simultaneous interactions between the CSP and the chiral analyte. This appears likely for the second-eluted enantiomers and somewhat less likely for the first-eluted enantiomers in this study.

On the bases of the thermodynamic data Fig. 4 clearly shows the existence of two subgroups of solutes. Trp (**1**), *erythro*-β-MeTrp (**2**), *threo*-β-MeTrp (**3**), CMP-Tic (**6**) and γ-phenyl-γ-butyrolactone (**7**) had smaller $-\Delta H^{o}$ and $-\Delta S^{o}$ values, while DNPyr-Trp (**6**) (and CBZ-Trp (**5**)) exhibited more negative values. In general, both the enthalpy change, $-\Delta H^{o}$, and entropy change, $-\Delta S^{o}$, for the transfer of analyte from the mobile phase to the CSP decreased with increasing MeOH content.

Trp (**1**) and β-MeTrp enantiomers (**2** and **3**) analogs in water-containing solutions were in zwitterionic form and capable of charge–charge (electrostatic) interactions with the CSP. For these solutes, the enthalpy change, $-\Delta H^{o}$, was below 30 kJ mol^{-1} and $-\Delta H^{o}$ values were greater in water-rich eluent (the only exception was D-Trp). Starting from a 0.1%TEAA–MeOH (80:20, v/v) mobile phase composition, $-\Delta H^{o}$ and k decreased with increasing MeOH concentration in the mobile phase (Fig. 4 and Table 1). This can be explained by a significant decrease in the hydrophobic interactions between each enantiomer and the CSP as the mobile phase becomes less polar. For analytes **1, 2** and **3,** a U-shaped curve of k as a function of MeOH content was observed; at high MeOH concentration, this phenomenon was due to the decreased solubility of the amino acids, the analyte favored the stationary phase.

Analytes **4** and **5** exhibited large enthalpy and entropy changes and these changes increased with higher concentrations of buffer (water) in the eluent, which is typical of a hydrophobic association between the analyte and the stationary phase. The large $-\Delta H^{o}$ and $-\Delta S^{o}$ values for analytes **4** and **5** can be attributed to the structures of the molecule. At pH 6.5, both analytes are negatively charged and the interaction with the positively charged CSP can explain the large $-\Delta H^{o}$ values. The *N*-carbobenzyloxy and *N*-(3,5-dinitro-2-pyridyl) substitution of **4** and **5** produced molecules that can participate in additional hydrophobic interactions, hydrogen bonding and steric interactions with the CSP and therefore exhibited large $-\Delta H^{o}$ and $-\Delta S^{o}$ values as compared with those for the Trp analogs (**1–3**). Slight decreases in $-\Delta H^{o}$ and $-\Delta S^{o}$ at highest water content (Fig. 4) can be explained by the hydrophobic effect caused by restructuring and reinforcement of the surrounding water molecules (iceberg formation) when a solute is introduced into a highly aqueous liquid [12]. The water molecules around the solute tend to be more highly structured to compensate for the molecular interactions broken by insertion of the solute, especially when the solute is nonpolar. The more positive entropy change, ΔS^{o}, at higher MeOH content might be attributed to different degrees of solvation in the mobile phase. The solvation of the enantiomers in a MeOH-rich eluent yielded a more ordered structure as compared with a water-rich mobile phase. Accordingly, the transition of the analyte from a MeOH-rich mobile phase to the CSP has a more positive entropy change (assuming that the analytes have the same freedom on the CSP).

For CMP-Tic (**6**) at pH 6.5, both the analyte and the CSP were positively charged, and therefore electrostatic attraction is unlikely. Retention and chiral recognition in the RP mode can be attributed to hydrophobic and steric interactions between the analyte and the CSP. CMP-Tic probably exhibited a favorable structure because of the nonplanar orientation of the two aromatic rings. The lack of a negatively charged group in the molecule eliminated the possibility of charge–charge attraction, and resulted in higher mass-transfer and adsorption–desorption kinetics. Therefore, an increase in resolution with increasing temperature and MeOH content could be observed.

γ-Phenyl-γ-butyrolactone, which is neutral, exhibited the smallest enthalpy change, $-\Delta H^{o} < 20$ kJ mol^{-1}. Its change as a function of MeOH concentration was similar to those for the other Trp analogs (**1–3**), except that at high MeOH content, $-T\ \Delta S^{o}$ overcompensates for the smaller $-\Delta H^{o}$. Therefore, k continuously decreased with increasing

MeOH content. Besides the hydrophobic interaction between γ-phenyl-γ-butyrolactone and the CSP, weak hydrogen bonding through the oxygen atoms must be taken into account in the retention mechanism.

The trends in the changes in $\Delta(\Delta H^{o})$ and $\Delta(\Delta S^{o})$ for the two subgroups of analytes are depicted in Table 4. For CBZ-Trp (**4**), DNPyr-Trp (**5**) and CMP-Tic (**6**) at higher MeOH content, both $-\Delta(\Delta H^{o})$ and $-\Delta(\Delta S^{o})$ were 2–3 times higher than those for other Trp analogs (**1, 2** and **3**) and for γ-phenyl-γ-butyrolactone (**7**) (Table 4). The more negative $\Delta(\Delta H^{o})$ value means that the interactions for these analytes are enthalpically favored. The more negative difference in entropy change, $\Delta(\Delta S^{o})$, for the series **4–6** can be explained by the fact that the difference in the degrees of freedom of these enantiomers on the CSP is larger. In **4** and **5,** the *N*-carbobenzyloxy and *N*-(3,5-dinitro-2-pyridyl) substitution resulted in a more hydrophobic character, together with an increased ability to undergo hydrogen bonding, and a stronger steric effect. For **6** the arrangement of the two ring systems in different planes very probably resulted in favorable steric interactions.

In **4** and **5,** strong hydrogen bonding groups (polar functional groups) were present and one possible conclusion was that hydrogen bonding, a strongly temperature-dependent phenomenon, was also involved in the enantioselective retention process [27]. The large $-\Delta(\Delta H^{o})$ values for **4** and **5** may originate from H-bonding. The more negative $\Delta(\Delta H^{o})$ with increasing MeOH content suggested that the enthalpic hydrogen bonding interaction between the analyte and the CSP was more pronounced as the MeOH content increased.

The Planck function $-\Delta(\Delta G^{o})/T$ versus $1/T$ for solutes **1–7** in all chromatographic modes could be fitted by straight lines. Fig. 5 depicts the plot of $-\Delta(\Delta G^{o})/T$ versus $1/T$ for analytes **1–7** in reversed-phase mode at 0.1% TEAA–MeOH mobile phase composition of 60:40 (v/v). Similar straight lines were obtained at other mobile phase compositions and also in the PO and NP modes. The correlation coefficient ranged between $0.991 < r^2 < 0.999$.

The difference in Gibbs free energy, $-\Delta(\Delta G^{o})$, versus MeOH concentration in the mobile phase

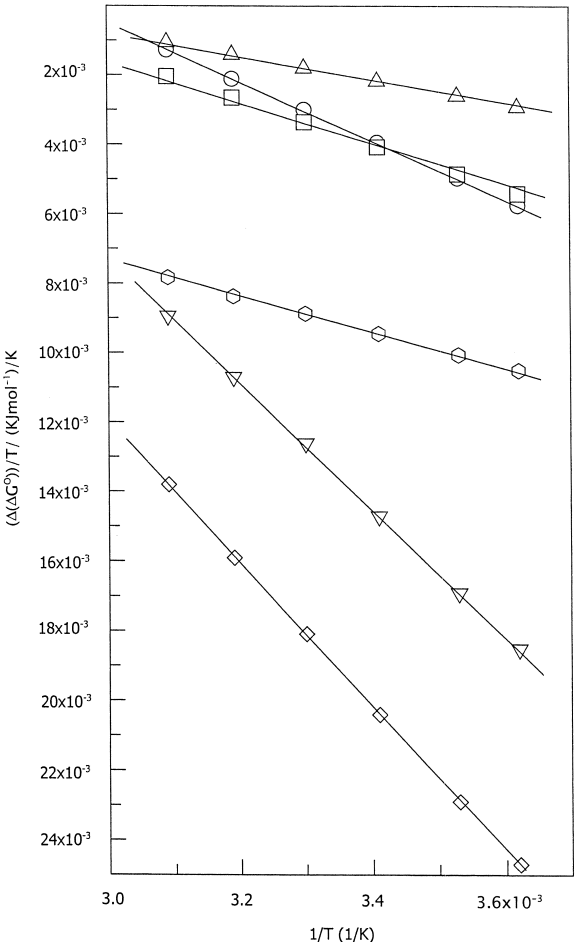

Fig. 5. Planck function $-\Delta(\Delta G^{o})/T$ versus $1/T$ for analytes **1–7** in reversed-phase mode at a mobile phase composition of 0.1% TEAA–MeOH (60:40, v/v). Chromatographic conditions: column, Chirobiotic R; flow-rate, 1 ml min^{-1}; detection, 205 nm; ○ Trp; □, *erythro*-β-MeTrp; △, *threo*-β-MeTrp; ▽, CBZ-Trp; ◇, DNPyr-Trp; ⬡, CMP-Tic; +, γ-phenyl-γ-butyrolactone; slopes, intercepts and correlation coefficients of the straight lines, Trp, $-7.7955x + 0.0226$, $r^2 = 0.9915$; *erythro*-β-MeTrp, $-5.9552x + 0.0161$, $r^2 = 0.9911$; *threo*-β-MeTrp, $-3.2626x + 0.0089$, $r^2 = 0.9928$; CBZ-Trp, $-16.666x + 0.0421$, $r^2 = 0.9918$; DNPyr-Trp, $-18.953x + 0.00441$, $r^2 = 0.9929$; CMP-Tic, $-4.6299x + 0.063$, $r^2 = 0.9934$; γ-phenyl-γ-butyrolactone, $-2.361x + 0.051$, $r^2 = 0.9921$.

(Fig. 6) clearly shows the existence of two subgroups of solutes. The first subgroup, analytes **1–3** and **7,** had smaller $-\Delta(\Delta G^{o})$ values, and analytes **4–6** had larger $-\Delta(\Delta G^{o})$ values. The Gibbs free

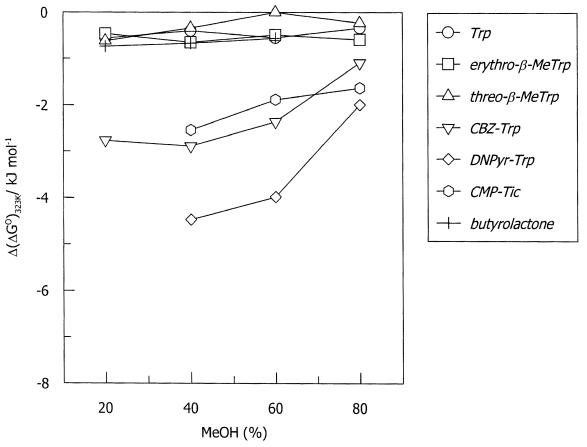

Fig. 6. Difference in Gibbs free energy, $\Delta(\Delta G^{o})$ between second and first-eluted enantiomers as a function of mobile phase composition, calculated at 323 K. Chromatographic conditions: column, Chirobiotic R; flow-rate, 1 ml min^{-1}; detection, 205 nm; mobile phase, aqueous 0.1% TEAA (pH 6.5)–MeOH (80:20, 60:40, 40:60, 20:80, v/v); ○, Trp; □, *erythro*-β-MeTrp; △, *threo*-β-MeTrp; ▽, CBZ-Trp; ◇, DNPyr-Trp; ⬡, CMP-Tic; +, γ-phenyl-γ-butyrolactone.

energy difference, which drives chiral recognition, was small for the first subgroup and resulted in poorer enantioselectivity, while the second subgroup with higher $-\Delta(\Delta G^{o})$ resulted in very high α factors.

3.6. Thermodynamic parameters in the polar-organic mode

The PO mode of enantiomeric separation was developed within the last 8 years. This mode is related to the NP mode, although the mobile phase contains no *n*-hexane or other nonpolar organic solvents. The main eluent components in this mode are MeCN and MeOH (with very small amounts of glacial HOAc and TEA). The main interactions seen in the PO mode are hydrogen bonding, π–π and steric interactions. To some extent, charge–charge interactions may occur.

The thermodynamic parameters obtained in the PO mode are given in Table 5. In all cases, the measured enthalpies, ΔH^{o}, were negative, indicating that the transfer of solute from the mobile phase to the sorption site is enthalpically favored. The entropy change, ΔS^{o}, exhibited both negative and positive values. However, as part of the retention process, molecules of strong solvents, which are sorbed on the stationary phase, are probably replaced by sorbed solute. The release of different amounts of polar solvents by the two enantiomers could make a positive contribution to $\Delta(\Delta S^{o})$. It was also characteristic that, for all analytes, the ΔH^{o} and ΔS^{o} values obtained in this mode were more positive than those in the RP mode.

The ΔH^{o} values were negative for all compounds tested in the PO mode. Positive ΔS^{o} values were observed for the β-MeTrp enantiomers. Since these ΔH^{o} and ΔS^{o} values compensated one another, the ΔG^{o} values for the Trp and β-MeTrp analogs were in a similar range, as in the RP analyses (data not shown), and resulted in similar retention profiles in both chromatographic systems.

For CBZ-Trp (**4**), DNPyr-Trp (**5**) and CMP-Tic (**6**), the ΔH^{o} and ΔS^{o} values were more negative than for **1–3**, but less negative than those obtained for these analytes in the RP mode. Therefore, the *k*, α and R_S values obtained for **4, 5** and **6** were smaller in the PO mode than in the RP mode. A probable explanation of this phenomenon for **4** and **5** may be that in PO mode charge–charge and hydrophobic interactions are negligible, while in the RP mode they predominate. CMP-Tic was positively charged under the RP conditions. Thus, hydrogen bonding, hydrophobic and steric interactions were important for retention and selectivity. In the PO mode, hydrogen bonding, dipolar interactions, steric interactions and π–π interactions were dominant.

The $\Delta(\Delta H^{o})$ and $\Delta(\Delta S^{o})$ values were more negative for **4–6** than for **1–3**. Analytes **4–6** were capable of additional π–π interactions. The $\Delta(\Delta G^{o})$ values of the *N*-blocked Trp analogs were more negative (especially for **6**) and higher α values were obtained. The selectivity for the enantiomers of CMP-Tic (**6**) in the PO mode was especially high ($\alpha > 10$) at all temperatures.

In the PO mode, an increase in R_S was observed with increasing temperature (except for CMP-Tic), but not always over the entire temperature range. This behavior can be explained by advantageous kinetic effects. The mass transfer and adsorption–desorption kinetics were probably more favorable in the PO mode as the temperature increased.

3.7. Thermodynamic parameters in the normal-phase mode

The ristocetin A CSP can also be used in the NP mode. The main associative interactions in the NP mode as in the PO mode, are hydrogen bonding, π–π complexation, dipole stacking, and steric repulsion.

Some of the solutes were not analyzable in the NP mode because of solubility factors. The thermodynamic parameters are presented in Table 5. The solutes had negative enthalpies and entropies of transfer. The more retained solute of each enantiomeric pair had the more negative ΔH^{o}, indicating stronger interactions with the CSP. The more retained enantiomer may form a stronger complex with CSP due to a greater number of points of interaction with the chiral surface. This could explain the negative ΔS^{o}.

For β-MeTrp enantiomers, somewhat larger $-\Delta H^{o}$ values were obtained in the NP mode than in PO mode and the ΔS^{o} values were also more negative. For CBZ-Trp (**4**) and DNPyr-Trp (**5**), the ΔH^{o} values were similar in both chromatographic systems, while the ΔS^{o} values were more positive in the NP mode. In these two chromatographic modes, ΔG^{o} and consequently the retention factors did not differ substantially. $\Delta(\Delta G^{o})$ was a measure of the differential enantioselective interactions between each enantiomer and this CSP. For the β-MeTrp enantiomers the $\Delta(\Delta G^{o})$ values were similar in both chromatographic modes, and therefore the α values also were similar. For CBZ-Trp (**4**) and DNPyr-Trp (**5**), the $\Delta(\Delta G^{o})$ values were more positive and this led to lower selectivity and poorer resolution in the NP mode.

γ-Phenyl-γ-butyrolactone (**7**), which exhibited the smallest $-\Delta H^{o}$ and $-\Delta S^{o}$ values in the RP mode, had relatively high $-\Delta H^{o}$ and $-\Delta S^{o}$ values in the NP mode. In the NP mode, besides the π–π interaction, the more temperature-dependent hydrogen bonding was probably the driving force in the enantioseparation. In an EtOH-rich Hex–EtOH = 20:80 (v/v) eluent, the enantiomers were not retained. In *n*-hexane-rich eluent, with Hex–EtOH (95:5, v/v), partial separation could be observed. The higher concentration of EtOH reduced the retention as a consequence of competitive hydrogen bonding for the CSP between the analyte and EtOH and therefore the hydrogen bonding of the analyte to the CSP weakened. At higher *n*-hexane content, (i) the interaction of EtOH with the CSP is less, and (ii) the hydrogen bonding interactions of the two enantiomers with the CSP were similar, while $\Delta(\Delta H^{o})$ and $\Delta(\Delta S^{o})$ were near zero. Therefore, very poor stereoselectivity and R_S values were obtained.

3.8. Enthalpy–entropy compensation

A further thermodynamic approach to the analysis of physicochemical data is enthalpy–entropy compensation [47]. The enthalpy–entropy compensation method was used in a study of hydrophobic interactions and separation mechanisms in RP-HPLC [48], and subsequently, in a study of enantioselective retention mechanism in GC [22]. Mathematically, enthalpy–entropy compensation can be expressed by the formula

$$\Delta H^{o} = \beta \Delta S^{o} + \Delta G^{o}_{\beta} \quad (3)$$

where ΔG^{o}_{β} is the Gibbs free energy of a physicochemical interaction at the compensation temperature, β (β and ΔG^{o}_{β} are constants). According to Eq. (3), when enthalpy–entropy compensation is observed for a group of compounds in a particular chemical transformation (or interaction in the case of chromatographic retention), all of the compounds have the same free energy change, ΔG^{o}_{β}, at temperature β. For example, if enthalpy–entropy compensation is observed in liquid or gas chromatography for a group of compounds, all the compounds will have the same net retention at the compensation temperature β, although their temperature dependences may differ. The similarity of the values for the compensation temperature suggests that the solutes are retained by essentially identical interaction mechanisms, and the compensation study is therefore a useful tool for comparing the retention mechanisms for different compounds. However, the results obtained with this method cannot be used alone: they can be misleading, due to the cumulative errors associated with the determination of enthalpy [49,50].

Fig. 7 shows enthalpy–entropy compensation plots (ΔH^{o} vs. ΔS^{o}) for the L- and D-enantiomers of **1–7** at four different mobile phase compositions on the

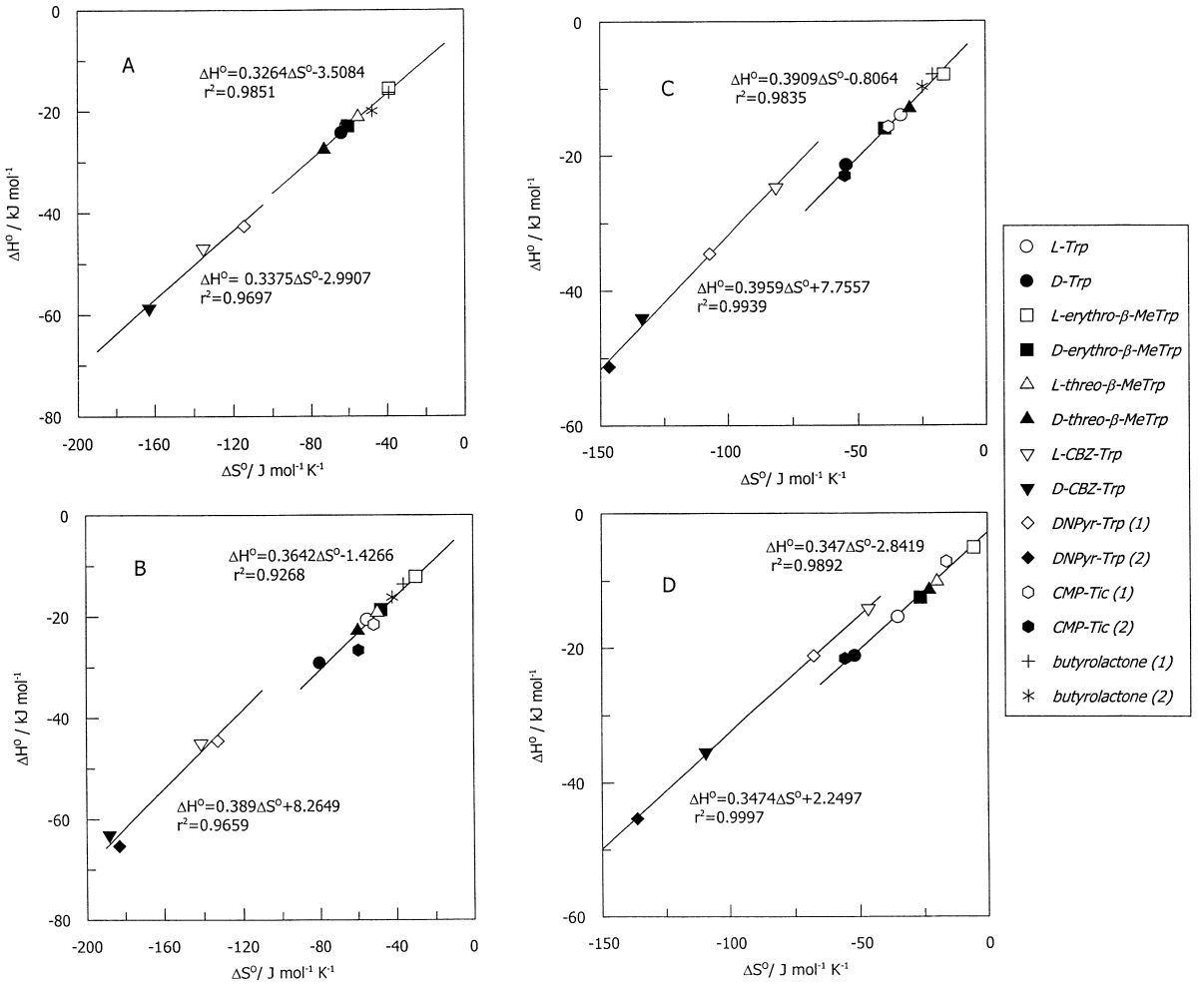

Fig. 7. Plots of enthalpy–entropy compensation for first and second-eluted enantiomers of analytes **1–7** at different mobile phase compositions Chromatographic conditions: column, Chirobiotic R; flow-rate, 1 ml min^{-1}; detection, 205 nm; mobile phase; (A) aqueous 0.1% TEAA (pH 6.5)–MeOH (80:20), (B) aqueous 0.1% TEAA (pH 6.5)–MeOH (60:40); (C) aqueous 0.1% TEAA (pH 6.5)–MeOH (40:60) and (D) aqueous 0.1% TEAA (pH 6.5)–MeOH (20:80, v/v); slopes, intercepts and correlation coefficients of the straight lines, (A) $\Delta H^{o} = 0.326 \times \Delta S^{o} - 3.508$, $r^2 = 0.9851$ and $\Delta H^{o} = 0.337 \times \Delta S^{o} - 2.991$, $r^2 = 0.9697$, (B) $\Delta H^{o} = 0.364 \times \Delta S^{o} - 1.426$, $r^2 = 0.9628$ and $\Delta H^{o} = 0.389 \times \Delta S^{o} + 8.264$, $r^2 = 0.9659$, (C) $\Delta H^{o} = 0.391 \times \Delta S^{o} - 0.8064$, $r^2 = 0.9835$ and $\Delta H^{o} = 0.396 \times \Delta S^{o} + 7.755$, $r^2 = 0.9939$, (D) $\Delta H^{o} = 0.347 \times \Delta S^{o} - 2.841$, $r^2 = 0.989$ and $\Delta H^{o} = 0.347 \times \Delta S^{o} + 2.249$, $r^2 = 0.9997$; open symbols, first-eluted enantiomer; filled symbols, second-eluted enantiomer; ○, ●, Trp; □, ■, *erythro*-β-MeTrp; △, ▲, *threo*-β-MeTrp; ▽, ▼, CBZ-Trp; ◇, ♦, DNPyr-Trp; ⬡, ⬢, CMP-Tic; +, ✷ γ-phenyl-γ-butyrolactone.

Chirobiotic R CSP. This degree of correlation can be considered adequate for verifying enthalpy–entropy compensation. The regression lines for the enantiomers at four different 0.1% TEAA–MeOH mobile phase ratios are given in the legend to Fig. 7.

It can be seen from these compensation data that the analytes in this study can be divided into two subgroups: (a) the Trp, β-MeTrp enantiomers, CMP-Tic and γ-phenyl-γ-butyrolactone belong in one group, (b) and the more bulky and the more hydrophobic CBZ-Trp and DNPyr-Trp belong in the other group. Within the groups, the compensation data (plots) for the L- and D-enantiomers are not significantly different from one another. This indicates

that the two enantiomers are retained via similar retention mechanisms. Change of the mobile phase conditions (i.e. 0.1% TEAA to MeOH) probably alters the retention mechanism of the compounds studied.

3.9. Retention mechanism on ristocetin A CSP

In the working pH range of the column (0.1% TEAA, pH 6.5), ristocetin A was positively charged and **1–6** existed in the zwitterionic form, or were negatively or positively charged. γ-Phenyl-γ-butyrolactone **7,** having no ionizable group, was neutral. Thus, the carboxylic acid moieties were in the anionic form $-COO^-$, and the primary amino groups were in the cationic form $-NH_3^+$. Earlier studies [1] showed that the ristocetin A ammonium group is the most available and logical site for the initial docking and enantioselective retention of compounds that contain an anionic moiety. Hydrophobic interactions may also be most important. Additional secondary interactions involve hydrogen bonding and steric interaction sites [1,2]. Any hindrance of these interactions weakens chiral recognition. The bulkiness and rigidity of the molecule can influence the interactions necessary for chiral recognition. In most cases, increasing rigidity and bulkiness improve the enantioselectivity on the ristocetin A CSP.

In the RP mode (at pH 6.5), Trp and β-MeTrp enantiomers were in zwitterionic form, and the charge–charge interaction, hydrophobic interaction, hydrogen bonding and steric interactions were the main driving forces in the separation. On *N*-carbobenzyloxy and *N*-(3,5-dinitro-2-pyridyl) substitution, the original Trp molecule became more hydrophobic, bulky and rigid and exhibited a negative charge at the working pH. Due to these complex effects in the RP mode, the molecules were strongly retained. The strong interaction with the CSP resulted in larger $-\Delta H^\circ$ and $-\Delta S^\circ$ values than obtained for Trp and β-MeTrp enantiomers. In the PO and NP modes, for all Trp analogs (**1–5**) the lack of electrostatic and hydrophobic interactions led to the retention being smaller, the separation poorer together with smaller $-\Delta H^\circ$ and $-\Delta S^\circ$ values.

In the positively charged CMP-Tic the two rings were situated in different planes, and groups were available for hydrogen bonding. The strong hydrogen bonding was accompanied by large $-\Delta H^\circ$ and $-\Delta S^\circ$ changes in RP and PO chromatographic systems. The presence of hydrophobic and other secondary interactions, (e.g. steric and π–π interactions) are probable.

γ-Phenyl-γ-butyrolactone was neutral and capable only of hydrophobic and other secondary interactions. On the basis of the thermodynamic parameters in the RP mode, hydrophobic and steric interactions were important, while in NP mode the more temperature-dependent hydrogen bonding and π–π stacking were the driving forces in the enantioseparation.

4. Conclusions

The effects of temperature on the retention demonstrated that the enantiomers of tryptophan, 1,2,3,4-tetrahydroisoquinoline and γ-phenyl-γ-butyrolactone analogs could be separated by using either subambient or elevated temperatures. Linear van't Hoff plots were observed in the studied temperature range, 276–323 K, and the apparent changes in enthalpy, ΔH°, entropy, ΔS°, and Gibbs free energy, ΔG°, were calculated. The values of the thermodynamic parameters depended on the structures of the compounds. In the case of carboxylic acid-type analogs, the main step in the chiral recognition and enantioselective retention on the ristocetin A-containing CSP probably is the charge–charge interaction between the carboxylate group and the ammonium group of the ristocetin A molecule, but for both charged and uncharged analytes, the hydrophobic interactions, hydrogen bonding, π–π complexation and steric interactions must also be considered. The thermodynamic data obtained support the proposed mechanism. The retention in the PO mode and NP modes tends to be less than in the RP mode. This was due to the lack of electrostatic and hydrophobic interactions in these modes. However, enantioselective retention is still possible due to hydrogen bonding, and dipolar and perhaps π–π interactions. Two distinct types of retention behavior are seen for this group of analytes.

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