



Investigations into the interaction thermodynamics of TRAP-related peptides with a temperature-responsive polymer-bonded porous silica stationary phase

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ABSTRACT

The interaction thermodynamics of the thrombin receptor agonistic peptide (TRAP-1), H-Ser-Phe-Leu-Leu-Arg-Asn-Pro-OH, and a set of alanine scan substitution peptides, have been investigated with an *n*-octadecylacrylic polymer-bonded porous silica (Sil-ODA₁₈) and water-acetonitrile mobile phases at temperatures ranging from 5 to 80 °C in 5 °C increments. The retention of these peptides on the Sil-ODA₁₈ stationary phase decreased as the water content in the mobile phase was lowered from 80% (v/v) to ca. 45% (v/v) and reached a minimum value for each peptide at a specific water-acetonitrile composition. Further decreases in the water content of the mobile phase led to increased retention. The magnitude of the changes in enthalpy of interaction, ΔH_{assoc}^0 , changes in entropy of interaction, ΔS_{assoc}^0 , and changes in heat capacity, ΔC_p^0 , were found to be dependent on the molecular properties of the mobile phase, the temperature, the structure/mobility of the stationary phase, and the conformation and solvation state of the peptides. With water-rich mobile phases, the retention behaviour of the TRAP analogues was dominated by enthalpic processes, consistent with the participation of strong hydrogen bonding effects, but became dominated by entropic effects with acetonitrile-rich mobile phases as the temperature was increased. These changes in the retention behaviour of these TRAP peptides are consistent with the generation of water or acetonitrile clusters in the mobile phase depending on the volume fractions of the organic solvent as the Sil-ODA₁₈ stationary phase transitions from its crystalline to its isotropic state.

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1. Introduction

The two models most widely employed to explain the retention behaviour of polar and non-polar analytes in reversed-phase liquid chromatography (RPLC) are the adsorption model [1,2] and the partition model [3–6]. The thermodynamic basis of the adsorption model in RPLC has been elaborated in terms of solvophobic theory by Horváth and coworkers [1]. This theory predicts that the retention of an analyte is largely controlled by the free energy changes associated with the formation in the bulk mobile phase of a cavity with dimensions of the analyte, and the generation of new water-water contacts when non-polar surface-accessible regions of the analyte interact with the immobilized non-polar chromatographic ligands attached to a stationary phase. In this solvation/

desolvation adsorption model, the structural re-organisation of relatively short (e.g. C3 to C8) *n*-alkyl ligands immobilized onto porous silica-based stationary phase has often been assumed to make only a small contribution to retention. However, as shown by Martire and Boehm [7] and other investigators [8–15], changes in the organisation of *n*-alkyl chains immobilized onto porous silica stationary phases can play an important role in determining retention behaviour, depending on the *n*-alkyl chain length and ligand density/surface coverage.

In order to accommodate these effects, Dill *et al.* [3–6], proposed a partition model based on the mean field statistical thermodynamic theory. In this partition model, analytes become embedded between the chains of non-polar *n*-alkyl ligands, such as *n*-octadecyl groups, immobilized onto the surface of a silica support material, rather than being adsorbed at the stationary phase/mobile phase interface. Accordingly, in this partition model, analyte retention with immobilized *n*-alkyl ligands depends *inter alia* upon the entropy of solvent mixing, the molecular conformation of the

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immobilized ligands and the physicochemical nature of the association of the analytes with these ligands [3].

Two principal predictions related to the impact of chain ordering of the stationary phase on analyte retention arise from the partition model [3,5]. Firstly, analytes are predicted to be preferentially distributed nearer to more mobile, and thus more exposed, regions of the ligand chains rather than confined to regions closer to the site of anchorage to the support material. This prediction has been confirmed by neutron scattering experiments with deuterated solvents, e.g. deuterated hexane and dioleoyllecithin bilayers [16]. Secondly, analytes are predicted to be increasingly expelled from the stationary phase as the immobilized ligand density approaches its maximum value. Sentell and Dorsey [17] have confirmed this prediction through measurement of the partition coefficients of naphthalene with a series of *n*-octadecylsilica stationary phases of the same surface density, and concluded that the chain organisation of the stationary phase played a major role in retention. Moreover, Cole *et al.* [18] have observed that at certain temperatures, the corresponding ΔH_{assoc}^0 and ΔS_{assoc}^0 values for benzene were more positive with low-density (less ordered) *n*-octadecylsilica stationary phases compared to high-density (more ordered) *n*-octadecylsilica stationary phases.

The extent of re-ordering of the immobilized ligands is therefore an important factor which must be taken into account to accommodate changes in the thermodynamic properties of analytes with reversed-phase chromatographic adsorbents. We and other investigators have previously shown that with temperature-responsive polymers immobilized onto porous silica or organic stationary phases the organisation of the polymer chains is an important factor leading to selectivity enhancement [19–28]. These stationary phases tend to form highly ordered crystalline states at low temperatures, e.g. below ca. 30 °C, but at higher temperatures, transition to less ordered isotropic states. Changes in selectivities observed for analytes with such temperature-responsive silica-based stationary phases in their crystalline and isotropic states have been found to be similar to the selectivity changes observed for high- and low-density *n*-octadecylsilica stationary phases [8–15,20,21], 4-(allyloxy)benzoyl-4-methoxy-phenyl silica stationary phases [29], mixed aminoalkyl/cholesterol-silica stationary phases [30] as well as cholesterol-modified open tubular capillary electrochromatography (OTCEC) fused-silica capillaries [31].

In this current study, this behaviour has been further investigated with a set of synthetic peptides related to the thrombin receptor agonistic peptide (TRAP-1), H-Ser-Phe-Leu-Leu-Arg-Asn-Pro-OH, separated at different temperatures with a comb-shaped polymeric *n*-octadecylacrylsilica (Sil-ODA₁₈) stationary phase in the presence of 20–80% (v/v) water-acetonitrile mobile phases containing 0.09% (v/v) trifluoroacetic acid. Changes in the retention and interaction thermodynamics of these TRAP peptides are discussed in terms of the transition of this stationary phase from its crystalline to isotropic state, conformational changes of the peptides and the formation of clusters of acetonitrile molecules at higher volume fractions of the organic solvent in the mobile phase.

2. Experimental

2.1. Chemicals and materials

HPLC grade acetonitrile (ACN) and methanol (MeOH) were obtained from Biolab Scientific Pty Ltd (Sydney, Australia), and trifluoroacetic acid (TFA) from Auspep Pty Ltd (Melbourne, Australia). Water was distilled and deionized with a Milli-Q system (Millipore, Bedford, MA, USA). The comb-shaped polymer (ODA₁₈) was prepared as described previously [20,32,33] by telomerization of octadecylacrylate with mercaptopropyltrimethoxysilane. The

polymer was then immobilized onto porous silica particles (5 μm, 120 Å, 295 m²g⁻¹) (YMC, Wilmington, NC, USA) to obtain the Sil-ODA₁₈ adsorbent which was then packed into stainless steel columns (4.6 × 150 mm) as reported previously [20]. The thrombin receptor agonistic peptide, TRAP-1 (H-Ser-Phe-Leu-Leu-Arg-Asn-Pro-OH), and its alanine-scan analogues, TRAP-2 (H-Ser-Ala-Leu-Leu-Arg-Asn-Pro-OH), TRAP-3 (H-Ser-Phe-Ala-Leu-Arg-Asn-Pro-OH), TRAP-4 (H-Ser-Phe-Leu-Ala-Arg-Asn-Pro-OH), TRAP-5 (H-Ser-Phe-Leu-Leu-Ala-Arg-Asn-Pro-OH), TRAP-6 (H-Ser-Phe-Leu-Leu-Arg-Ala-Pro-OH), were synthesized by Fmoc/Boc SPPS (solid phase peptide synthesis) methods [34,35].

2.2. Instrumentation

Chromatographic measurements were performed using a Waters 600/486 HPLC system, a 71P WISP auto-injector with the Millennium software (MA, USA). Temperature was controlled by immersing the columns into a Cole Palmer Polystat (Chicago, Illinois, USA) heating circulator. Chromatographic peak profiles were monitored at 215 nm. Retention measurements were performed using 5–80% (v/v) water/acetonitrile containing 0.09% (v/v) TFA, delivered at a flow rate of 1 mL min⁻¹ and at temperatures of 5–80 °C in 5 °C increments. Bulk solvents were filtered through 0.45 μm polysulphone membrane and degassed by sparging with helium. The injected volume of each peptide, (1 mg/mL dissolved in water containing 0.09% TFA) was 10 μL. The retention factor (*k*) was determined from $k = (t_e - t_0)/t_0$, where *t_e* and *t₀* are the retention times of the analyte and sodium nitrate used as the void volume marker, respectively. All data points were derived from at least triplicate measurements, with the *t_e* values of replicates varying by less than 1%. The various thermodynamic and extra-thermodynamic parameters were calculated using the Hephæstus software developed in this laboratory [36], with experimental data collated as Excel (Microsoft) spreadsheets that contained imbedded macros corresponding to the mathematical expressions for the different thermodynamic parameters. Statistical analyses and nonlinear regression analyses involved the SigmaPlot 13 program (Systat Software Inc., San Jose, California). The standard deviations of replicates were smaller than the size of the data points shown as Figs. 1–4.

3. Results and discussion

3.1. Properties of the comb-shaped polymer immobilized silica, Sil-ODA_n

The crystalline to isotropic phase transition of the immobilized polymeric *n*-octadecyl-acrylsilica (Sil-ODA₁₈) stationary phase has been previously characterized by differential scanning calorimetry (DSC) and selectivity profiling with a variety of low molecular weight aromatic analytes [19–21,32,37–39]. The transition midpoint temperature (*T_m*) as determined by DSC procedures was near to 40 °C for a mobile phase composed of 0.09% (v/v) TFA-20% (v/v) water/methanol [20,21]. With the acetonitrile-based mobile phases employed in the current investigations, the *T_m* values, as determined by DSC measurements, were found to be lower, e.g. for a 0.09% (v/v) TFA-20% (v/v) water/acetonitrile mobile phase the *T_m* was near 35 °C. This phase transition temperature, *T_m*, shifting to higher values when the water content of the mobile phase was increased.

Depending on the temperature and composition of the water-acetonitrile mobile phase, three discrete regions of phase transition were observed, namely a crystalline, liquid-crystalline and isotropic state with the phase organisation decreasing in the order of crystalline > liquid-crystalline > isotropic as the temperature

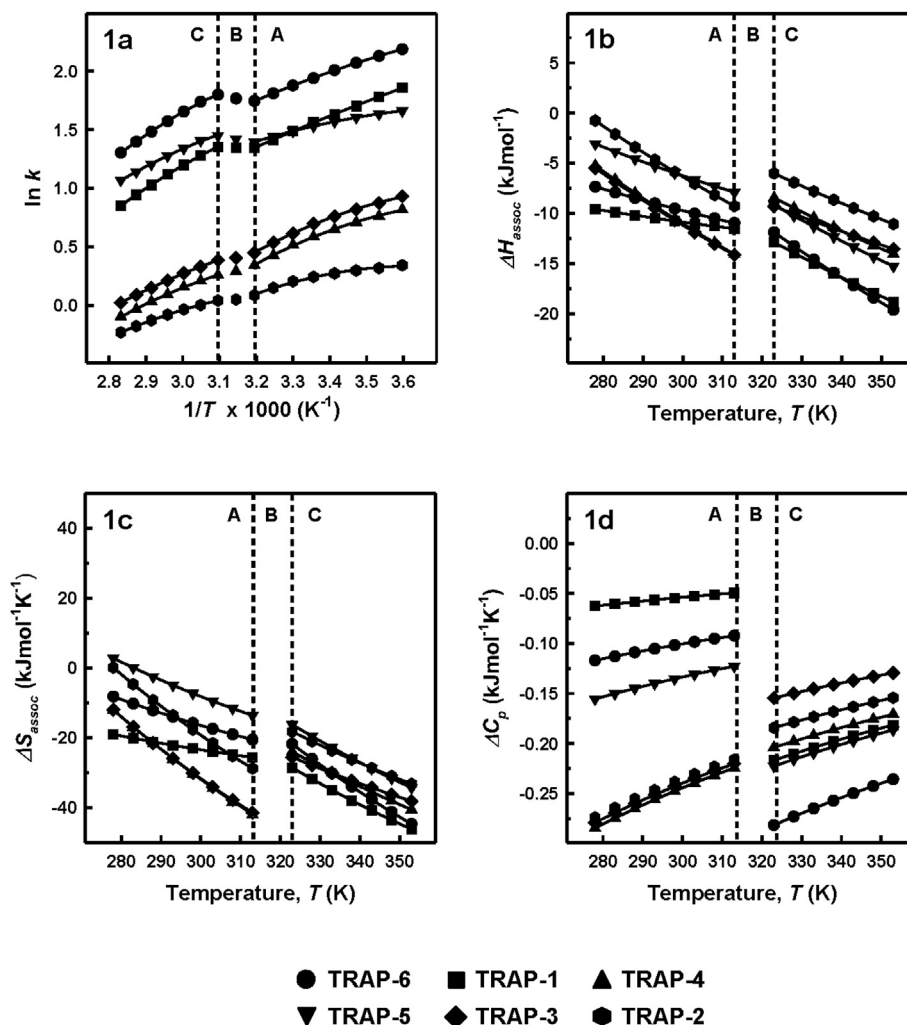


Fig. 1. Plots of the logarithmic retention factor $\ln k$ versus $1/T$ (1a), enthalpy ΔH_{assoc}^0 versus T (1b), entropy ΔS_{assoc}^0 versus T (1c) and heat capacity ΔC_p^0 (1d) versus T for the interaction of TRAP-1 and its alanine-scan peptide analogues with the comb-shaped polymer immobilized silica (Sil-ODA₁₈ stationary phase) in a water-acetonitrile (80:20, v/v) system containing 0.09% TFA with the experimental data fitted to a second order polynomial function in region A ($T = 278\text{--}313\text{ K}$) and a second order polynomial fit in region C ($T = 323\text{--}353\text{ K}$).

was increased. The liquid–crystalline phase represents a specific state between the crystalline (most ordered) and isotropic (least ordered) states. The liquid–crystalline state formed as the crystalline phase commenced to melt at temperatures above about 30 °C with 0.09% (v/v) TFA–20% (v/v) water–acetonitrile or 20% (v/v) water/methanol. In this mesophasic state [20,21], the ratio of liquid–crystalline to crystalline phase of the Sil-ODA₁₈ bonded polymer increases as the temperature was increased from 30 °C to 40 °C, whilst above 40 °C, the ratio of isotropic to liquid–crystalline phase increased.

3.2. Theoretical considerations governing the retention behaviour, the characteristics of the van't Hoff plots and the interaction thermodynamics of the TRAP peptides with the Sil-ODA₁₈ stationary phase using water-organic solvent mobile phases

For a steady state chromatographic interaction between a TRAP peptide and the immobilized non-polar ligands of the Sil-ODA₁₈ stationary phase, the extent of retention can be evaluated from the ratio of the bound to free peptide concentration [40,41]. This ratio can be expressed in terms of the retention factor (k), and takes the form of Eqn (1):

$$k = \frac{n_S}{n_M} \times \frac{V_S}{V_M} = K_{assoc} \times \frac{V_S}{V_M} = K_{assoc} \times \Phi \quad (1)$$

where n_S and n_M are the number of moles of the peptide in the bound and free states respectively, K_{assoc} is the equilibrium binding constant and Φ is the phase ratio of the system, defined as the ratio V_S/V_M , where V_S and V_M are the volume of the stationary phase and the volume of the solvent in the system, respectively. Several approaches for the measurement of the phase ratio, Φ , can be found in the literature [42], whereby changes in the contribution of the phase ratio to the retention factor, k , can be accommodated [36]. Because of the relatively small observed changes in the magnitude of the dead time, t_0 , and thus the phase ratio, Φ , of the column, due to very minor changes in the volume of the ligand solvation layer compared to the fixed volume of the supporting silica particle, the observed substantial variations in $\ln k$ as a function of T as T was changed for a fixed mobile-phase composition must reflect the changes in K_{assoc} associated with analyte–ligand interactions arising from the reorganization of the stationary-phase surface from the crystalline to the liquid–crystalline and finally to the isotropic state. The dependency of k on temperature can be expressed in terms of

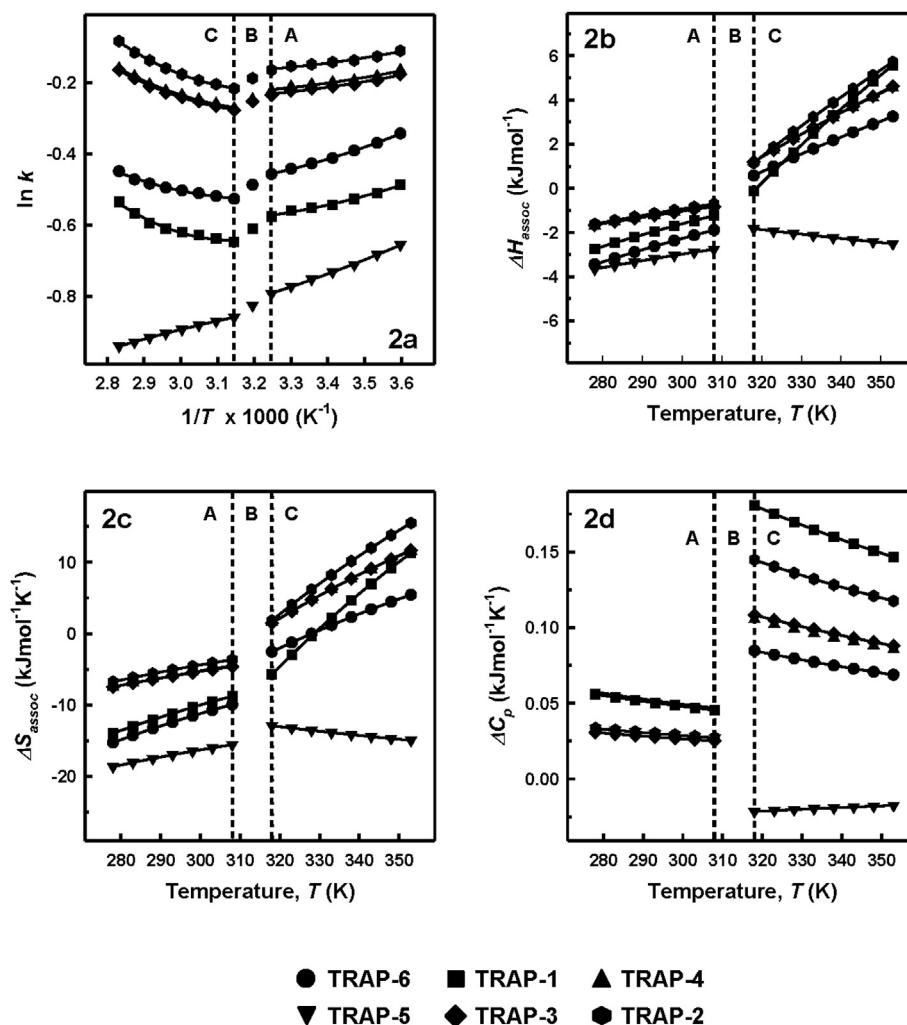


Fig. 2. Plots of the logarithmic retention factor $\ln k$ versus $1/T$ (2a), enthalpy ΔH_{assoc}^0 versus T (2b), entropy ΔS_{assoc}^0 versus T (2c) and heat capacity ΔC_p^0 (2d) versus T for the interaction of TRAP-1 and its alanine-scan peptide analogues with the comb-shaped polymer immobilized silica (Sil-ODA₁₈ stationary phase) in a water-acetonitrile (30:70, v/v) system containing 0.09% TFA with the experimental data fitted to a second order polynomial function in region A ($T=278-308$ K) and a second order polynomial fit in region C ($T=318-353$ K).

the fundamental thermodynamic relationship (Eqn (2)):

$$\ln k = -\frac{\Delta H_{assoc}^0}{RT} + \frac{\Delta S_{assoc}^0}{R} + \ln \phi \quad (2)$$

where ΔH_{assoc}^0 and ΔS_{assoc}^0 are the changes in enthalpy and entropy associated with the peptide-non-polar ligand interactions, R is the universal gas constant and T is the absolute temperature in degree Kelvin. Plots of $\ln k$ versus $1/T$ (van't Hoff plots) thus provide a facile approach to determine ΔH_{assoc}^0 and ΔS_{assoc}^0 , and the corresponding ΔC_p^0 , of the system. When the interaction process is isothermic, linear van't Hoff plots are anticipated, with the change in heat capacity, ΔC_p^0 , equalling zero [36,43]. However, for homothermic or heterothermic processes whereby $\Delta C_p^0 \neq 0$ and is a function of T [36,43], curvilinear van't Hoff plots are anticipated. For such non-linear van't Hoff plots, the dependence of $\ln k$ on T can be approximated by the following relationship:

$$\ln k = b_{(0)} + \frac{b_{(1)}}{T} + \frac{b_{(2)}}{T^2} + \ln \phi \quad (3)$$

Hence, from Eqns (1) and (2), the change in enthalpy, ΔH_{assoc}^0 , can be expressed as:

$$\Delta H_{assoc}^0 = -R \left[b_{(1)} + \frac{2b_{(2)}}{T} \right] \quad (4)$$

whilst the change in entropy, ΔS_{assoc}^0 , is given by:

$$\Delta S_{assoc}^0 = R \left[b_{(0)} - \frac{b_{(2)}}{T^2} \right] \quad (5)$$

and the change in heat capacity, ΔC_p^0 , is given by:

$$\Delta C_p^0 = R \left[\frac{2b_{(2)}}{T^2} \right] \quad (6)$$

where $b_{(0)}$, $b_{(1)}$ and $b_{(2)}$ are steri-molar structural parameters specific for each peptide.

As evident from Figs. 1a, 2a and 3a, non-linear $\ln k$ versus $1/T$ plots were obtained for the various TRAP-related peptides at different volume fractions, ϕ -values, of the organic solvent using water/acetonitrile mixtures containing 0.09% (v/v) TFA over the

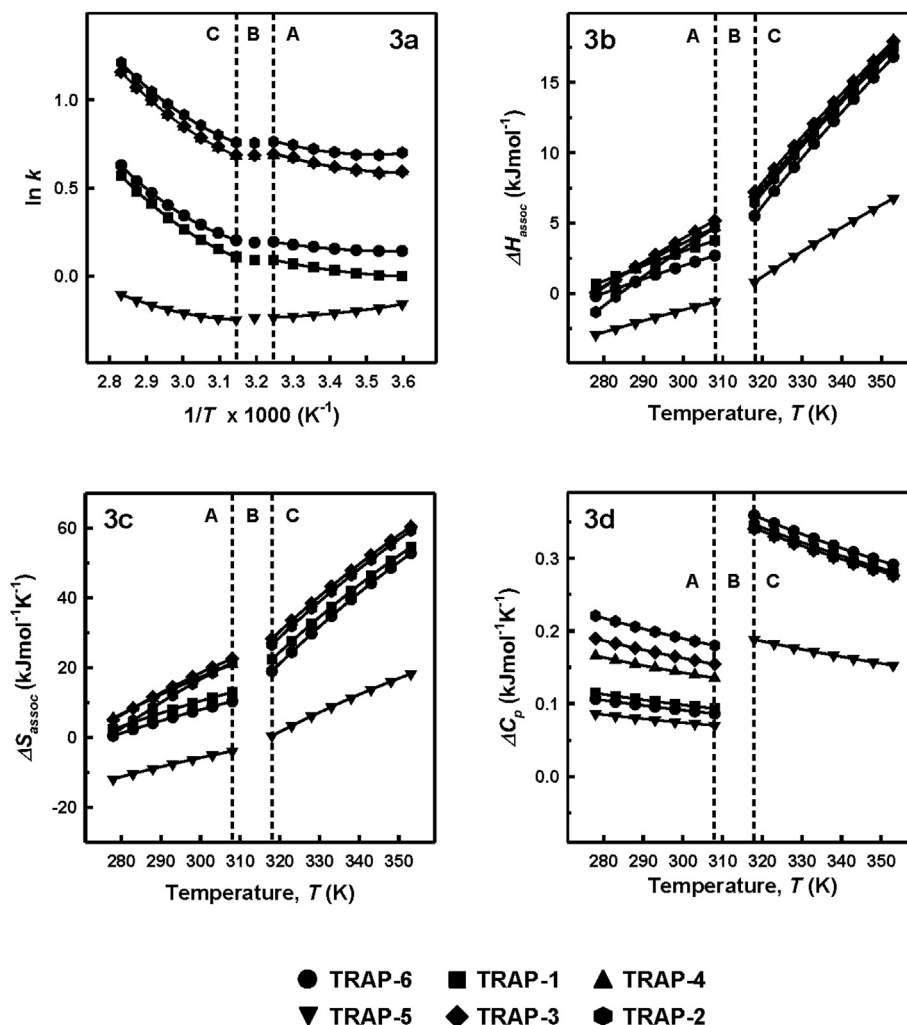


Fig. 3. Retention factor $\ln k$ versus $1/T$ (3a), enthalpy $\Delta H^0_{\text{assoc}}$ versus T (3b), entropy $\Delta S^0_{\text{assoc}}$ versus T (3c) and heat capacity ΔC_p^0 (3d) versus T for the interaction of TRAP-1 and its alanine-scan peptide analogues with a comb-shaped polymer immobilized silica (Sil-ODA₁₈ stationary phase) stationary phase in a water-acetonitrile (20:80, v/v) containing 0.09% TFA with the experimental data fitted to a second order polynomial function in region A ($T = 278\text{--}308\text{ K}$) and a second order polynomial fit in region C ($T = 318\text{--}353\text{ K}$).

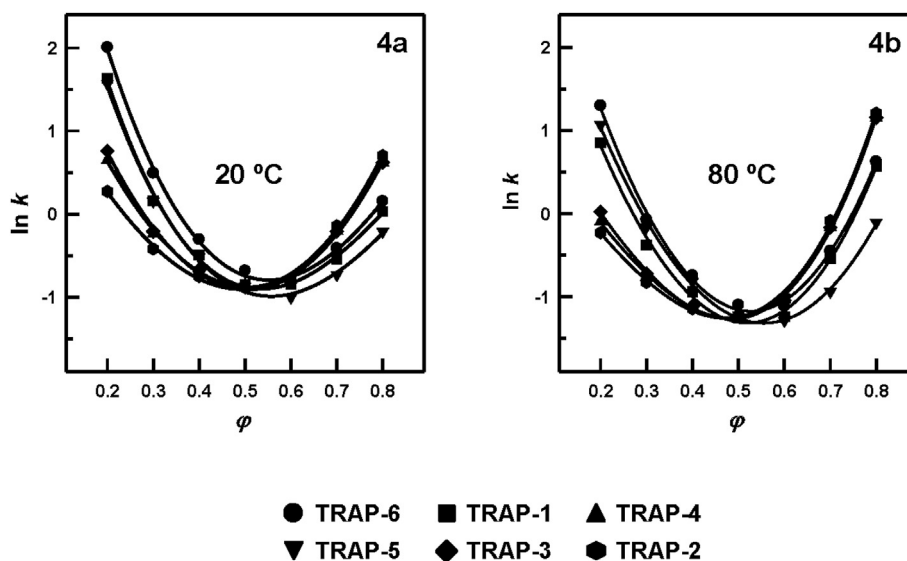


Fig. 4. Logarithmic retention factor, $\ln k$, versus ϕ , the volume fraction of acetonitrile in the aqueous organic mobile phase for the Alanine-scan TRAP peptides at 293 K (4a) and at 353 K (4b).

temperature range 278–358 K. In order to encompass a global retention trajectory linking $\ln k$ versus $1/T$ that also simultaneously accommodated the crystalline, liquid–crystalline and isotropic phase states over the studied temperature range, derivation of the thermodynamic parameters, ΔH_{assoc}^0 , ΔS_{assoc}^0 and ΔC_p^0 , required the use of fifth-order regression analyses in order to fit the experimental data sets for each TRAP peptide (data not shown). Alternatively, if the crystalline and isotropic states were considered as two independent phase states, then the experimental data corresponding to the $\ln k$ versus $1/T$ dependencies encompassing three discrete regions, namely A, B and C (from lowest to highest temperature) and representing the crystalline (A), liquid–crystalline (B) and isotropic (C) states of the Sil-ODA₁₈ stationary phase, could be fitted to a second order polynomial function (Eqn (3)) with high correlation coefficient R^2 values and small residual χ^2 values. The related values of ΔH_{assoc}^0 , ΔS_{assoc}^0 and ΔC_p^0 for the TRAP-related peptides could then be derived, according to Eqns (4)–(6). Moreover, by evaluating the experimental data encompassing the temperature region A (corresponding to the crystalline state, $5^\circ\text{C} \leq T \leq 35^\circ\text{C}$), and the temperature region C (corresponding to the isotropic state, $45^\circ\text{C} \leq T \leq 85^\circ\text{C}$), in terms of second-order $\ln k$ versus $1/T$ dependencies, the values of ΔH_{assoc}^0 , ΔS_{assoc}^0 and ΔC_p^0 so obtained for these TRAP-related peptides for these two discrete temperature ranges were found to be consistent with previous DSC studies. Due to the narrow temperature range and limited data available for the liquid–crystalline state (region B) the corresponding thermodynamic parameters for this B-state were not explored further.

3.3. Retention, enthalpy and entropy changes of the TRAP peptides with the Sil-ODA₁₈ stationary phase and mobile phase composed of 0.09% (v/v) TFA–80% (v/v) water/acetonitrile

Previously, Boysen *et al.* [35] have investigated the interaction of several TRAP related peptides with a conventional *n*-octylsilica stationary phase (*i.e.*, Zorbax 300SB–C8) in order to gain insights into the conformational properties of these peptides in RPLC environments. Molecular modelling and associated energy minimization studies indicated that all of the TRAP peptides used in the current study could adopt compact globular shape in water–acetonitrile mixtures at room temperatures.

The elution order of several of the TRAP peptides, namely TRAP-2 ($X_{hydro} = 14.24$) < TRAP-4 ($X_{hydro} = 16.91$) < TRAP-3 ($X_{hydro} = 16.91$) < TRAP-1 ($X_{hydro} = 20.86$) < TRAP-5 ($X_{hydro} = 22.22$) < TRAP-6 ($X_{hydro} = 24.75$) (Fig. 1a, values in parenthesis correspond to their calculated relative hydrophobicities, X_{hydro}) from the Sil-ODA₁₈ stationary phase in its isotropic state (region C) with a 0.09% (v/v) TFA–80% (v/v) water/acetonitrile mobile phase followed the same order as observed previously with a Zorbax 300SB–C8 column under similar elution conditions [35]. In contrast, TRAP-1 eluted after TRAP-5 when the Sil-ODA₁₈ stationary phase existed in its crystalline state (region A). The selectivities for the six TRAP analogues separated in the crystalline state of Sil-ODA₁₈ were similar to those found with polymeric stationary phase materials, respectively [19–21]. All of the TRAP peptides showed a decrease in retention with increasing temperature when the Sil-ODA₁₈ stationary phase was in the crystalline state (region A). Upon transition to the isotropic state with increasing temperature, initially small increases in retention were observed for TRAP-1, TRAP-5 and TRAP-6, whilst decreases in retention were evident for TRAP-2, TRAP-3 and TRAP-4. At higher temperatures, the retention for all TRAP peptides with the Sil-ODA₁₈ stationary phase in the isotropic state (region C) then decreased.

3.4. Retention, enthalpy and entropy changes of the TRAP peptides with the Sil-ODA₁₈ stationary phase with mobile phases comprising 0.09% (v/v) TFA–30% (v/v) or 20% (v/v) water/acetonitrile

Compared to the results obtained with a 0.09% (v/v) TFA–80% (v/v) water/acetonitrile mobile phase system, different elution orders were observed for the TRAP peptides with the Sil-ODA₁₈ stationary phase when either 0.09% (v/v) TFA 30% (v/v) or 0.09% (v/v) TFA 20% (v/v) water/acetonitrile was used as the mobile phase (Figs. 2a and 3a). For example, whilst TRAP-2 eluted first with the 0.09% (v/v) TFA 80% (v/v) water/acetonitrile mobile phase, this peptide eluted last with both the 0.09% (v/v) TFA 30% (v/v) and the 0.09% (v/v) TFA 20% (v/v) water/acetonitrile mobile phases. Furthermore, TRAP-2, TRAP-3 and TRAP-4 eluted before TRAP-1, TRAP-5 and TRAP-6 with the 0.09% (v/v) TFA 80% (v/v) water/acetonitrile mobile phase, but they eluted later with either the 0.09% (v/v) TFA 30% (v/v) or 20% (v/v) water/acetonitrile mobile phase. Peptides TRAP-1, TRAP-2 and TRAP-3, TRAP-4 and TRAP-6 showed decreased retention with increasing temperature when the Sil-ODA₁₈ stationary phase was in the crystalline state (region A) with 0.09% (v/v) TFA 30% (v/v) water/acetonitrile mobile phase, whilst when the Sil-ODA₁₈ stationary phase was in the isotropic state (region C) they showed increased retention with increasing temperature (Fig. 2a). In contrast, a decrease in retention with increasing temperature was evident for TRAP-5 for the Sil-ODA₁₈ stationary phase in the isotropic state. If the water content of the mobile phase was reduced further from 30% (v/v) to 20% (v/v) water/acetonitrile, all peptides except TRAP-5 showed increased retention with increasing temperature for the Sil-ODA₁₈ stationary phase in both the crystalline and isotropic states (Fig. 3a). A small decrease in retention with increasing temperature was observed for TRAP-5 for the Sil-ODA₁₈ stationary phase in the crystalline state (region A).

Moreover, the sign and magnitude of the derived ΔH_{assoc}^0 and ΔS_{assoc}^0 thermodynamic parameters for all of the TRAP analogues were negative or close to zero with the 0.09% (v/v) TFA 30% (v/v) water/acetonitrile mobile phase for the Sil-ODA₁₈ stationary phase in the crystalline state (region A), and except for TRAP-5 became positive with further increases in temperature for the Sil-ODA₁₈ stationary phase in the isotropic state (region C) (Fig. 2b and c). However, the negative ΔH_{assoc}^0 and ΔS_{assoc}^0 values of TRAP-5 became even more negative with increasing temperature when the Sil-ODA₁₈ stationary phase was in the isotropic state. The trends in the ΔH_{assoc}^0 versus T and ΔS_{assoc}^0 versus T plots for these peptides (except for TRAP-5) when the Sil-ODA₁₈ stationary phase was in the isotropic state with a 0.09% (v/v) TFA 30% (v/v) water/acetonitrile mobile phase were thus opposite of those observed with a 0.09% (v/v) TFA 80% (v/v) water/acetonitrile mobile phase. In addition, the ΔH_{assoc}^0 and ΔS_{assoc}^0 values for these peptides (except TRAP-5) in the 0.09% (v/v) TFA 20% (v/v) water/acetonitrile (Fig. 3b and c) were positive or close to zero at temperatures when the Sil-ODA₁₈ stationary phase was in the crystalline state, and rapidly increased with increasing temperature. Here, the strong temperature dependencies of ΔH_{assoc}^0 and ΔS_{assoc}^0 on T indicate that hydrogen bonding associated with peptide–water interactions do not solely provide an adequate explanation for the changed retention of these peptides with the Sil-ODA₁₈ stationary phase and mobile phase systems of low water content.

3.5. Evaluation of heat capacity changes of the TRAP peptides with the Sil-ODA₁₈ stationary phase in 0.09% (v/v) TFA 80% (v/v) water/acetonitrile mobile phase

The calculated accessible surface areas, ΔA_{tot} (\AA^2) [17] of the different TRAP peptides in their globular (G) and extended (E) conformations are: TRAP-1 (917.0 G, 1015.2 E), TRAP-2 (855.9 G,

923.9 E), TRAP-3 (883.4G, 964.7 E), TRAP-4 (883.4 G, 964.7 E), TRAP-5 (848.5 G, 913.1 E) and TRAP-6 (882.9 G, 964.0 E), respectively, e.g., the accessible surface areas of all TRAP peptides were slightly larger in their conformationally more extended states. The heat capacity of transfer of an analyte into water have been shown to be linearly proportional to the accessible surface area of nonpolar solutes [44–46], with the heat capacity for weakly hydrophobic analytes decreasing with increasing temperature [45]. In the present study, negative ΔC_p^0 values were obtained for all TRAP peptides using the 0.09% (v/v) TFA 80% (v/v) water/acetonitrile mobile phase when the Sil-ODA₁₈ stationary phase was in the crystalline state (region A), but became less negative with increasing temperature when this sorbent was in the isotropic phase (region C) (Fig. 1d).

Previous studies have shown that the transfer of nonpolar analytes from water to a lipid bilayer is associated with large and negative ΔC_p^0 values [47–49]. Large negative heat capacity changes ($\Delta C_p^0 \ll 0$) are the hallmark of processes that are driven by hydrophobic effects associated with the transfer of nonpolar analytes from water to the non-polar bilayer [50]. Vailaya and Horváth observed negative heat capacity changes for dansyl-derivatives of amino acids at low temperature in hydrophobic interaction chromatography (HIC), with the ΔC_p^0 decreasing in magnitude with increasing temperature [51]. The observed trends for the positive ΔC_p^0 values which decreased with increasing temperature in region A and region C, respectively, of the Sil-ODA₁₈ stationary phase with the TRAP analogues with the 0.09% (v/v) TFA 20% (v/v) water-acetonitrile mobile phase corroborate further the hypothesis that entropic effects increasingly contributed to the retention process of these peptides with the Sil-ODA₁₈ stationary phase when acetonitrile-rich mobile phases are used.

Spolar et al. have observed that the heat capacity increment upon unfolding of peptides/proteins is affected by two factors: the hydration of nonpolar groups, which produce positive ΔC_p^0 values at room temperature and which decrease with increasing temperature, and hydration of the polar groups, for which ΔC_p^0 is negative and decrease in magnitude with increasing temperature [37]. However, the contribution from polar groups is usually smaller (in absolute value) than that of the nonpolar groups, thus, the overall heat capacity change upon unfolding of peptides/proteins is usually observed to be positive. It is clear that the respective trends in the changes in the magnitudes of the thermodynamic parameters, ΔH_{assoc}^0 , ΔS_{assoc}^0 and ΔC_p^0 upon transfer of TRAP analogues from mobile phases of different acetonitrile contents onto the Sil-ODA₁₈ stationary phase and as the temperature was increased was associated with a transition of each peptide from a compact to a more extended structure.

3.6. Thermodynamic characterization of the interaction of the TRAP analogues with the Sil-ODA₁₈ stationary phase

Mechanistically, at least three processes can be proposed to occur when the TRAP analogues interact with the Sil-ODA₁₈ stationary phase at different temperatures in the presence of mobile phases of different acetonitrile content, namely (a) changes in the organisation of the stationary phase ligands; (b) changes in the conformation of the individual peptides, and (c) changes in the solvational status of each peptide in the bulk mobile phase and at the stationary phase-mobile phase interface. The above results indicate that the changes in the ΔH_{assoc}^0 , ΔS_{assoc}^0 and ΔC_p^0 parameters with temperature were highly dependent upon the solvent compositions, its solution structure and the organisational state of the stationary phase. When the binding of charged molecules to a hydrophobic surface occurs with negative ΔH_{assoc}^0 values, which become even more negative in magnitude with increasing temperature, this behaviour has been classified by Seeling and Ganz

[47] as an enthalpy-driven process. According to this scenario, the loss of peptide solvational enthalpy would be largely compensated by the gain in peptide dehydration enthalpy upon interaction with a non-polar stationary phase. Vailaya and Horváth [51] have observed negative ΔH_{assoc}^0 values at 25 °C for dansyl derivatives of amino acids in hydrophobic interaction chromatographic (HIC) measurements, with the ΔH_{assoc}^0 values becoming more negative with increasing temperature. The temperature dependence of ΔH_{assoc}^0 for the interaction of the TRAP peptides with the Sil-ODA₁₈ stationary phase with the 0.09% (v/v) TFA 80% (v/v) water/acetonitrile mobile phase system employed in this current study followed a similar trend. Thus, as evident from Fig. 1b, the enthalpy changes (ΔH_{assoc}^0) for all TRAP peptides were negative for both the crystalline and isotropic states (region A and region C) of the Sil-ODA₁₈ stationary phase with the 0.09% (v/v) TFA 80% (v/v) water/acetonitrile mobile phase and became more negative with increasing temperatures. However, the magnitudes and slopes of the ΔH_{assoc}^0 versus T dependencies were different prior to and following the transition of the Sil-ODA₁₈ stationary phase from its crystalline to isotropic state. These changes in retention behaviour are consistent with significant changes in hydrogen bonding associated with peptide-water and water-water contacts in the bulk mobile phase upon interaction of the peptides with the non-polar stationary phase in the presence of this mobile phase of high water content.

The observed increase in the positive ΔH_{assoc}^0 values for the TRAP analogues with increasing temperature with acetonitrile-rich mobile phases suggests that under these conditions the interaction of the TRAP analogues with this stationary phase becomes enthalpically less favourable as the Sil-ODA₁₈ stationary phase transitioned from its crystalline to isotropic state. Moreover, the increase in the magnitude of the positive ΔS_{assoc}^0 values with increasing temperature indicates a reduced level of entropic repulsion of the TRAP analogues as the mobility of the polymer chains of this stationary phase progressively increase with increasing temperature, a conclusion consistent with the interphase theory of retention [5,6,52]. Therefore, the retention of TRAP peptides with the Sil-ODA_n stationary phase with the 0.09% (v/v) TFA 30% (v/v) or 0.09% (v/v) TFA 20% (v/v) water/acetonitrile mobile phase systems became dominated by entropic processes with decreasing water content in the mobile phase and also with increasing temperature as the stationary phase ligands transition from the crystalline to the isotropic state.

An additional factor has also to be considered with this chromatographic system. The enthalpy of hydration associated with nonpolar groups of peptides in their globular state has been shown to be negative below room temperature, e.g. <298 K, but becomes positive at higher temperatures, whilst it is positive for polar groups of peptides at low temperature but negative at temperature above 298 K. For most experimental temperature ranges, the enthalpy of transfer for nonpolar groups of a peptide in a globular conformation will dominate, resulting overall in the enthalpy of transfer being negative below room temperature, but becoming positive with increasing temperature when the peptide transitions to a less globular/more extended state [53]. In RPLC systems with water-rich mobile phases, an opposite process, i.e., desolvation of nonpolar groups of a peptide, occurs upon interaction with the stationary phase, with the enthalpy changes occurring upon transition of the peptide to a more extended state following a trajectory in the opposite direction, i.e., become more negative with increasing temperature.

The changes in the ΔH_{assoc}^0 values for the TRAP analogues separated with the Sil-ODA₁₈ stationary phase using the 0.09% (v/v) TFA 80% (v/v) water/acetonitrile mobile phase system (Fig. 1b) are consistent with the above conclusions, e.g. the interaction of the

TRAP analogues in their more unfolded state becoming energetically more favourable with increasing temperature. Privalov et al. have observed that upon unfolding of a protein, the entropies of hydration of both nonpolar and polar groups were negative at low temperature, but change in different directions with increasing temperature [54]. By analogy, in the present investigation the changes of $\Delta S_{\text{assoc}}^0$ with temperature for the TRAP analogues separated with the Sil-ODA₁₈ stationary phase with the 0.09% (v/v) TFA 80% (v/v) water/acetonitrile mobile phase (Fig. 1c) are consistent with a retention process that involves desolvation of the nonpolar groups of the TRAP analogues and reduction in the number of peptide-water hydrogen bonds upon interacting with the Sil-ODA₁₈ stationary phase when mobile phases of very high water content are employed. Moreover, the negative ΔC_p^0 values associated with the interaction of the TRAP analogues with the Sil-ODA₁₈ stationary phase became positive with increasing temperature. This observation supports the hypothesis that at higher temperatures above 40 °C, hydrophobic driving forces dominates the retention of the TRAP analogues with the Sil-ODA₁₈ stationary phase when the 0.09% (v/v) TFA 80% (v/v) water/acetonitrile mobile phase is employed.

DeVido et al. [55] have previously observed negative $\Delta H_{\text{assoc}}^0$ and $\Delta S_{\text{assoc}}^0$ values for amino acids in RP-HPLC experiments using *n*-alkyl grafted stationary phases. In this study, the negative entropy was attributed to chain entropy effects upon the amino acid analytes attempting to interdigitate with the non-polar chains of the immobilized ligand. Partitioning of these analytes into these sorbents was observed to become entropically less favourable when the bonded chain density was increased, but was partially compensated by an energetically more favourable large negative enthalpy change arising from the tight contact between the ligands and the solutes. This partition process was envisioned to shift from enthalpy-driven to entropy-driven with increasing temperature. We have previously reported that the $\Delta H_{\text{assoc}}^0$ and $\Delta S_{\text{assoc}}^0$ values were negative for alkanes and cycloalkanes separated with the Sil-ODA₁₈ stationary phase in the crystalline state with a 0.09% (v/v) TFA 30% (v/v) water/methanol mobile phase at temperatures below 303 K, but these thermodynamic parameters became positive with increasing temperature [19]. A similar behaviour occurs with the Sil-ODA₁₈ stationary phase forming a highly ordered structure in the crystalline state at temperatures below 303 K (30 °C), but with increasing temperature adopts a less ordered structure on transition from the crystalline to the isotropic state [19–21]. The calculated entropy changes for the various TRAP analogues are also consistent with these structural changes of the Sil-ODA₁₈ stationary phase with the retention of these peptides by the Sil-ODA₁₈ stationary phase shifted from enthalpy-driven to entropy-driven as the Sil-ODA₁₈ stationary phase changed from its crystalline to the isotropic state with increasing temperature and organic solvent content in the mobile phase.

3.7. Evaluation of the origin of the unusual retention behaviour of the TRAP analogues with the Sil-ODA₁₈ stationary phase

As noted above, the retention behaviour of the TRAP analogues with the Sil-ODA₁₈ stationary phase can be attributed to (at least) three different phenomena as the temperature is increased in the presence of water-acetonitrile mobile phases of different organic solvent content, namely re-organisation of the immobilized ligands as the temperature is increased, conformational changes with the peptides, and formation of relatively short lived solvent structures in the mobile phase as the volume fraction, ϕ , of acetonitrile is varied. The $\ln k$ versus ϕ plots of TRAP analogues with the Sil-ODA₁₈ stationary phase were U-shaped between 20% (v/v) and 80% (v/v) acetonitrile in the aqueous organic mobile phase at both 20 and 80 °C, indicative of multiple mechanism for the TRAP-Sil-ODA₁₈

interactions (Fig. 4a and b). The TRAP-1, TRAP-5 and TRAP-6 showed the lowest $\ln k$ values with the 0.09% (v/v) TFA 60% (v/v) water/acetonitrile mobile phase, whereas TRAP-2, TRAP-3 and TRAP-4 reached their minimal value with a 0.09% (v/v) TFA 50% (v/v) water/acetonitrile mobile phase. As discussed above, the retention behaviour of the TRAP peptides with the Sil-ODA₁₈ stationary phase is consistent with a shift from an enthalpy-driven to an entropy-driven process as the content of water in the water-acetonitrile mixture was reduced. Furthermore, both the $\Delta H_{\text{assoc}}^0$ and $\Delta S_{\text{assoc}}^0$ became even more negative with increasing temperature in a water-rich mobile phase system with the stationary phase in both its crystalline and the isotropic states, whereas these parameters became positive with increasing temperature in acetonitrile-rich mobile phases (Fig. 1b, c, 2b, 2c, 3b and 3c). These findings are consistent with the solution structure of the mobile phase playing a significant role in the retention of the TRAP analogues with the Sil-ODA₁₈ stationary phase.

In addition, the selectivity of the Sil-ODA₁₈ stationary phase towards TRAP-1 and TRAP-5 changed on transition of this stationary phase from its crystalline to the isotropic state with a 0.09% (v/v) TFA 80% (v/v) water/acetonitrile mobile phase (Fig. 1a). Similar variations in selectivity have previously been reported for various low molecular weight organic molecules with the Sil-ODA₁₈ stationary phase under similar mobile phase conditions [19–21]. It can therefore be concluded that the Sil-ODA₁₈ stationary phase in its crystalline state compared to its isotropic state interacted more strongly with TRAP-5 than with TRAP-1, resulting in a gradual cross-over (reversal) of elution order for TRAP-5 and TRAP-1 as evident from the $\ln k$ versus temperature plots. In addition, the retention of TRAP-5 in the isotropic temperature range (i.e., 318–353 K) decreased with increasing temperature with the 0.09% (v/v) TFA 30% (v/v) water/acetonitrile mobile phase system, whereas the retention increased for the other TRAP analogues. The $\Delta H_{\text{assoc}}^0$ and $\Delta S_{\text{assoc}}^0$ data showed that the retention of TRAP-5 with the Sil-ODA₁₈ stationary phase in the isotropic state with the 0.09% (v/v) TFA 30% (v/v) water/acetonitrile mobile phase system was enthalpy-dominated whilst it was entropy-dominated for the other TRAP analogues (Fig. 2b and c). The main structural differences between TRAP-5 and the other TRAP analogues is that the basic amino acid, Arg, was replaced by Ala in TRAP-5. The retention behaviour of TRAP-5 correlates with the absence of dipolar and hydrogen bonding characteristics due to the replacement of the guanidinyll group of Arg⁵ in the TRAP sequence. Moreover, replacement of the TRAP Arg⁵ with Ala resulted in a smaller accessible surface area for TRAP-5 both in its globular and more extended conformational states.

With water-rich mobile phases, TRAP-2, TRAP-3 and TRAP-4 eluted earlier than TRAP-6, TRAP-5 and TRAP-1, whereas a reversal of this elution order was observed with acetonitrile-rich mobile phases, i.e. TRAP-6, TRAP-5 and TRAP-1 eluted earlier than TRAP-2, TRAP-3 and TRAP-4 (Figs. 1a, 2a and 3a). These peptides become conformationally more extended at higher temperatures with increasing acetonitrile content in the mobile phase. In its more extended conformational state, TRAP-1 has the largest accessible surface area (1015.20 Å²), followed by TRAP-6 (964.0 Å²), TRAP-3 (964.7 Å²), TRAP-4 (964.7 Å²), TRAP-2 (923.9 Å²) and TRAP-5 (913.1 Å²). However, the relative hydrophobicity is highest for TRAP-6 ($X_{\text{hydr}} = 24.75$), followed by TRAP-5 ($X_{\text{hydr}} = 22.22$), TRAP-1 ($X_{\text{hydr}} = 20.86$), TRAP-3 ($X_{\text{hydr}} = 16.91$), TRAP-4 ($X_{\text{hydr}} = 16.91$) and then TRAP-2 ($X_{\text{hydr}} = 14.24$). Therefore, the reversal of the elution order for these TRAP analogues with mobile phases of higher acetonitrile content is related to the combined effects of changes in the higher order structure of the Sil-ODA₁₈ stationary phase and changes in the solution structure of the mobile phase at the molecular level.

For separations governed solely by solvophobic effects, the transfer of analyte molecules into water is considered to be entropically driven, with ΔS_{assoc}^0 negative at room temperature and decreases in magnitude with increasing temperature. Cole et al. have concluded that such solvophobic effects represent the driving force for reversed-phase separations, as proposed also by Horváth and coworkers [1,2], since it accounts for analyte retention when water-rich mobile phases are employed, but suggested that the solvophobic effect did not adequately encompass the retention behaviour for other systems [18]. Cole et al. [18] also observed that at any given temperature the values of ΔH_{assoc}^0 and ΔS_{assoc}^0 for organic molecules were smaller in magnitude when *n*-octadecylsilica stationary phases of high bonding density ($4.07 \mu\text{mol}/\text{m}^2$) were employed compared to stationary phases of low bonding density ($2.39 \mu\text{mol}/\text{m}^2$), indicating in relative terms stronger entropic expulsion of analytes occurred from the stationary phases of high bonded density, but was nevertheless still an energetically favourable process. In this investigation, the sign and magnitude of ΔS_{assoc}^0 for the TRAP analogues at any given temperature depended upon the polymer chain entropy as well as the hydrophobic entropy of the analytes. Because the Sil-ODA₁₈ stationary phase undergoes a transition from an ordered crystalline state to a disordered isotropic state with increasing temperature, this transition results in an increase in accessibility of the stationary phase ligands for interactions with the TRAP peptides. The ordered structure of the Sil-ODA₁₈ stationary phase as well as the hydrogen bond network of the peptides with water in the mobile phase will be further disrupted as the water content of the mobile phase is decreased. With the 80% (v/v) water/acetonitrile mobile phase system, the decrease in negative ΔH_{assoc}^0 and ΔS_{assoc}^0 with increasing temperature was thus much larger for TRAP-2, TRAP-3 and TRAP-4 than for TRAP-1, TRAP-5 and TRAP-6 (Fig. 1b and c). These peptides also show a selectivity reversal in terms of the dependence of ΔH_{assoc}^0 and ΔS_{assoc}^0 on temperature upon increasing the acetonitrile content of the mobile phase systems (Fig. 1b and c, 2b, 2c, 3b and 3c).

Moreover, the ΔC_p^0 values of the TRAP analogues, which were positive but decreased slightly over the temperature range from 5 to 80 °C with the water-rich mobile phase, were negative with acetonitrile-rich mobile phases but became significantly less negative with increasing temperature in both regions A and C, respectively (Figs. 3d and 1d). The small decrease in ΔC_p^0 values with increasing temperature with water-rich mobile phases is indicative of an increase in nonpolar surface area exposed by the polymer surface. In contrast, the increasingly less negative ΔC_p^0 values for the TRAP analogues upon increasing the temperature with an acetonitrile-rich mobile phase system are consistent with contributions from the heat capacity changes arising from processes in addition to the structural re-organisation of the polymer chains of the Sil-ODA₁₈ stationary phase. A considerable body of research [56–60] has shown that acetonitrile and water molecules can form inhomogeneous mixtures at the molecular level although on the macroscopic scale their mixtures are homogeneous. In particular, clusters of acetonitrile molecules can form when the volume fraction φ reaches about 0.3 and a significant change in water structure occurs with regard to water-water hydrogen bond networks and analyte-water solvation. In the case of the TRAP analogues, conformational changes will be preceded by the disruption of these intra- and inter-molecular interactions, as the peptide chains gain conformation freedom with the more negative enthalpy and entropy of hydration of the non-polar groups of the peptide offset by reorganization of the hydrogen-bonding network of water [53,61]. By analogy to the work of Wimley et al. [48], it can be concluded that the polymer chains of the Sil-ODA₁₈ stationary phase will also gain conformational freedom upon decreasing the

water content in the mobile phase and also by increasing the temperature (i.e., upon changing from the crystalline to the isotropic state). These hydrogen bonding effects will be partly counter-balanced, resulting in increases in enthalpy and entropy, leading to reduction in the strength of the hydrophobic driving force due to decreasing the water content in the mobile phase.

Based on the retention and thermodynamic data, it can be envisioned that at a particular mobile phase and temperature condition, the overall ΔH_{assoc}^0 and ΔS_{assoc}^0 changes that occur upon interaction of the TRAP analogues with the Sil-ODA₁₈ stationary phase can be related to: (i) disruption of intramolecular interactions (increase in ΔH_{intra}^0 and ΔS_{intra}^0), (ii) hydration of nonpolar groups (decrease in ΔH_{hydr}^0 and ΔS_{hydr}^0), (iii) dehydration upon contact with the polymer phase (increase in ΔH_{dehydr}^0 and ΔS_{dehydr}^0), and (iv) due to the polymer chain mobility (increase in ΔH_{mobil}^0 and ΔS_{mobil}^0 with increasing chain ordering/mobility). In an aqueous-rich mobile phase system (i.e., 80% (v/v) water/acetonitrile), peptide-water hydrogen bond-based effects probably dominate the overall enthalpy and entropy changes, resulting in negative ΔH_{assoc}^0 and ΔS_{assoc}^0 values. Disruption of these intramolecular interactions upon unfolding of the peptides is expected to be partly offset by an increase in the dipole mediated solvation effects of the aprotic organic solvent. However, the TRAP analogues are more likely to be adsorbed onto the Sil-ODA₁₈ stationary phase in its crystalline state under this experimental condition. Therefore, the partial dehydration of the peptides upon adsorption onto the polymer surface result in overall negative changes in ΔH_{assoc}^0 and ΔS_{assoc}^0 . In contrast, with acetonitrile-rich mobile phase systems, the dipole mediated solvation effects of the aprotic organic solvent will probably be over-compensated by disruption of intramolecular interactions of the peptides and dehydration effects. Although the peptides were expected to exhibit higher contact areas in their interactions with the polymer chains of the Sil-ODA₁₈ stationary phase with acetonitrile rich mobile phases, these polymer chain also gain more mobility or configurational freedom. This increase in chain mobility will contribute to increases in both ΔH_{assoc}^0 and ΔS_{assoc}^0 . As a consequence, the ΔH_{assoc}^0 and ΔS_{assoc}^0 values become positive or close to zero with mobile phases containing a high percentage of acetonitrile, and become more positive with increasing temperature.

For separations of non-polar analytes with conventional porous *n*-alkylsilicas, solvophobic theory predicts that entropy changes will be positive at low temperatures but become negative with increasing temperature [51]. Consistent with this conclusion, Cole et al. [18] obtained positive ΔS_{assoc}^0 values for benzene with *n*-octadecylsilica columns using 95% water/1-propanol mobile phase, which decreased in magnitude with increasing temperature. In contrast, the retention of the TRAP peptides with the Sil-ODA₁₈ stationary phase in 0.09% (v/v) TFA 80% (v/v) water/acetonitrile mobile phase can be characterized as an enthalpically favourable process, whereby ΔH_{assoc}^0 becomes increasingly negative as the temperature is increased, whilst, as the content of water in the mobile phase was decreased, particularly when the volume fraction of the organic solvent was above $\varphi = 0.5$. The retention of TRAP analogues with the Sil-ODA₁₈ stationary phase progressively became entropically dominated.

4. Conclusions

This study has documented that the interaction thermodynamics of TRAP analogues with the Sil-ODA₁₈ stationary phase under different chromatographic conditions depend upon the hydrogen bonding properties of the mobile phase, the ordering/mobility of stationary phase and the conformational status of the peptides. These effects collectively determined the sign and the

magnitude of the thermodynamic parameters ΔH_{assoc}^0 and ΔS_{assoc}^0 . Retention of TRAP analogues with the Sil-ODA₁₈ stationary phase in both its crystalline and isotropic states with water-rich mobile phases was dominated by enthalpic effects, but with acetonitrile-rich mobile phases by entropic effects. The results also indicated that with water-rich mobile phases, retention of the TRAP analogues more likely occurred by adsorption effects, whereas with acetonitrile-rich mobile phases, at least partial embedment of the TRAP analogues into the sorbent chain structure can occur, and such embedment increased with increasing acetonitrile content when the Sil-ODA₁₈ stationary phase was in its isotropic state.

Conflict of interest

The authors declare no conflict of interest.

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List of symbols

$b_{(0)}, b_{(1)}, b_{(2)}, b_{(3)}$	Coefficients for the polynomial dependency on $\ln k$ versus $1/T$
c_i	Mole fraction of displacing solvent
ΔC_p^0	Change in the heat capacity for the association of the polypeptide P_i with the non-polar ligates
ΔG_{assoc}^0	Change in Gibbs free energy due to the association of the polypeptide P_i with the non-polar ligates
ΔH_{assoc}^0	Change in the enthalpy due to the association of the polypeptide P_i with the non-polar ligates
ΔS_{assoc}^0	Change in the entropy due to the association of the polypeptide P_i with the non-polar ligates
φ	Volume fraction of organic solvent in binary water-solvent mixture
Φ	Phase ratio of the chromatographic system
k	Retention factor $k = (t_e - t_0)/t_e$
K_{assoc}	Equilibrium binding constant
$\ln k$	Logarithm of the retention factor, $\ln k$
$\ln k_0$	Value of $\ln k$ when $c_i \rightarrow 0$, or $\varphi \rightarrow 0$
r^2	Correlation coefficient
R	Gas constant
t_0	Retention time of non-interacting solute
t_e	Retention time of a polypeptide P_i
T	Temperature in degrees Kelvin

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