Temperature effects on a doubly tethered diproline chiral stationary phase: Hold-up volume, enantioselectivity and robustness

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Abstract

Effect of temperature on hold-up volume, enantioselectivity and robustness of a novel doubly tethered diproline chiral stationary phase (CSP1) was studied. In-column end-capping of residual silanol was utilized as a tool to exhibit *in situ* change of CSP1. The hold-up volume marker, 1,3,5-tri-*tert*-butylbenzene (TTBB), was observed to be weakly retained (<1 second) on a 5 cm x 4.6 mm chiral column, and its retention time was changed with the carrier solvent and column temperature. The apparent thermodynamic parameters of TTBB indicated an enthalpy-driven retention process with the hexane/isopropanol (IPA) mobile phase, while an entropy-driven process with the hexane/methyl *tert*-butyl ether (MTBE) mobile phase. The $\Delta\Delta H$ and $\Delta\Delta S$ values of chiral separation for the four probes including 1,1'-bi-2-naphthol and warfarin were negative on CSP1. Nonlinear van't Hoff plots were observed for some analytes before and after the end-capping treatment. Depending on compound, end-capping strengthened or weakened the enantioseparation. Moreover, the enantioselectivity of CSP1 was shown to be robust by testing with heating-cooling cycles and step-temperature programs.

Introduction

In high performance liquid chromatography (HPLC), most methods are carried out at around 15 - 50˚C (typically at room temperature), with the exception of just a few methods employing high temperatures (Guillarme and Heinisch 2005). Because temperature as a parameter can furnish useful information on chromatographic processes, influence of temperature on enantioselective separation has been extensively studied (Török *et al*. 2006, Choi *et al*. 2007, Weng *et al*. 2008, Berthod 2009). Investigations by variable temperature conditions have contributed to a better mechanistic understanding of enantioselectivity in HPLC.

To implement these studies, the hold-up volume (V_0) of the column, an indispensable variable, has been shown valuable for calculating chromatographic and thermodynamic parameters. For example, error in estimating hold-up volume may cause significant errors in nonlinearly chromatographic parameters, e.g., coefficient of the equilibrium isotherm (Samuelsson *et al*. 2008, Gritti and Guiochon 2009). Hold-up volume can be measured by un-retained neutral markers, isotopic labeled solute, organic and inorganic salts, pycnometry, minor disturbance, and homologous series methods (Rimmer *et al*. 2002). Widely used 'un-retained' markers in normal-phase HPLC include TTBB. In 1983, Koller *et al*. first suggested TTBB as a hold-up volume marker for triacetylcellulose based CSPs with ethanol-water mobile phases. In 1991, Pirkle and Welch evaluated TTBB as a hold-up volume marker under normal phase conditions for brush-type CSPs. Since then, TTBB has been utilized as a primary hold-up volume marker in HPLC. However, its slight retentions on Chiralcel OJ and Chiralpak IA columns have been qualitatively reported (Pirkle and Welch 1991; Lao and Gan 2007, 2008). As quantitative TTBB retention time has been somewhat unknown until now, concern should be given regarding the certainty of measured hold-up volumes.

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End-capping of residual silanol has become an important step for preparation of HPLC stationary phases. There are typically three end-capping of residual silanol temperatures/procedures: at room temperature (Pirkle and Readnour 1991, Pirkle *et al*. 1992, Hyun *et al*. 1996), elevated temperature at > = 60°C or refluxing in solvent (Jinno *et al*. 1989, Yang *et al*. 2006), and high-temperature silylation at >250°C (Sudo 1996). HPLC stationary phase refluxing with silanization reagent, e.g., trimethylchlorosilane or hexamethyldisilazane (HDMS), in toluene/pyridine is a common method of end-capping for HPLC columns, especially for a reversed-phase C18 column that is non-enantioselective; therefore, there are no configuration and/or racemization issues involved. In preparation of chiral stationary phases (CSPs), e.g., brush-type (or Pirkle-type), additional consideration should be given to end-capping procedures to avoid making any unexpected changes on the chiral selector (Pirkle and Readnour 1991, Oliveros *et al*. 1992). End-capping of residual silanol by passing HDMS reagent through the column at room temperature was used to evaluate CSP over a long period of time (Hyun *et al*. 1996, Wolf and Pirkle 1998). Besides its effectiveness and convenience, this method has the potential to change the environment of chiral selectors *in situ* to minimize extra or unexpected change on the CSP. Consequently, information directly linked to interactions among the chiral selector, analyte, and silanol may emerge, which may facilitate understanding of the separation process. On the other hand, if the column in evaluation and application comes from a commercial source, further treatment of the column may alter its performance and is usually not encouraged. Accordingly, many types of CSPs have no reports on evaluation of potential effects from end-capping treatment.

The robustness test evaluates the capacity of an analytical procedure to remain unaffected by small, but deliberate variations in method parameters and provides an assurance of its reliability during normal usage (Dejaegher and Heyden 2007). Reasonably, a robustness test for a novel CSP is particularly important to evaluation; currently, no consistent standard robustness test method is available. Cyclic and step-temperature programs, having been exerted on polysaccharide-based CSPs, could be used as a robustness test to examine reproducibility and flexibility in CSPs (Lao and Gan 2006, Wang *et al*. 2008b).

Several new oligoproline CSPs without end-capping have exhibited powerful chiral recognition ability (Huang *et al*. 2006, Lao and Gan 2009b). Among these CSPs, a doubly tethered diproline CSP (CSP1) has been previously prepared and evaluated (Figure 1; Lao and Gan 2009a). For CSP1, the goals of present study are three-fold: 1) evaluate TTBB's behavior, 2) assess the effect of temperature on enantioselectivity associating to end-capping, and 3) characterize enantioseparation robustness via cyclic and step-temperature programs.

METHODS

Chemicals and Apparatus

TTBB, 5,5',6,6',7,7',8,8'-octahydro(1,1'binaphthalene)-2,2'-diol (**1**) , 1,1'-bi-2-naphthol (**2**), warfarin (**3**), coumachlor (**4**), and fipronil (**5**) as well as HDMS were purchased from Aldrich (Milwaukee, WI; Figure 2). All HPLC-grade solvents were purchased from Fisher (Pittsburgh, PA). An Agilent 1100 HPLC systems (Wilmington, DE) consisting of a vacuum degasser, a quaternary pump, an autosampler, a thermostatic column compartment, and a multiple wavelength detector was used to evaluate the column. The sign of rotation for the resolved enantiomers was determined by an in-line advanced laser polarimeter (PDR-Chiral, Lake Park, FL) connected after the multiple wavelength detector. Chromatographic signal was recorded by Agilent Chemstation.

Chiral Column and Chromatographic Measurements

The CSP1 column (Figure 1) was prepared and evaluated as in Lao and Gan 2009a. The chiral selector loading was 0.28 mmol/g on Kromasil HPLC grade spherical silica gel (particle size = 5 µm; pore size = 100 Å, and surface area = 298 m^2/g; Eka Chemical, CT). The chiral column (50 mm x

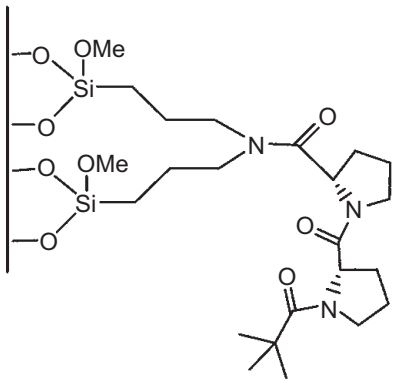

Figure 1. CSP1 structure.

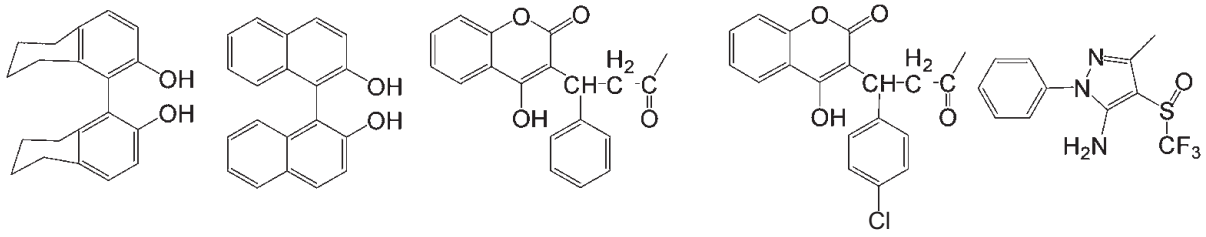

Figure 2. Structure of probe molecules used in this study.

4.6 mm of modular column; Isolation Technologies, Hopedale, MA) was prepared by the standard slurry packing method. The end-capping procedure followed documented method (Pirkle and Readnour 1991, Pirkle *et al*. 1992). The residual silanol groups were end-capped by passing through a solution of 3 ml of HDMS in 80 ml of dichloromethane at a flow rate of 1 ml/min. The CSP was further washed with 100 ml of DCM, followed by 100 ml of methanol, 30 ml of IPA to remove NH_3, and unreacted HDMS (Pirkle and Terfloth 1995). Usually to characterize end-capping, elemental analysis is conducted. However, because *in situ* end-capping may provide unique information on the CSP, elemental analysis was not conducted in this study.

In this study, the chromatographic conditions were kept constant with a 1.0 ml/minute flow rate, 254 nm detection wavelength, and 20 µl injection volume. Analytes **1** through **5** were dissolved in mobile phase at 600 mg/kg for analysis. The column thermostat of the HPLC instrument controlled the column temperature at 15 - 50°C. The extra column time of the HPLC system measured with a zero-volume connector in place of the column, with 0.07 minutes subtracted from the measured retention time. The column temperature was changed step-wise over the range of 15 - 50°C. At each temperature (15, 25, 35, 45 and 50°C), the column was equilibrated with the mobile phase for 1 hour before the sample injection. The heating-cooling temperature program included temperature steps of 15, 25, 35, 45, 50, 45, 35, 25 and 15°C. The step-temperature program consisted of temperature points at 15, 50, 15 and 50°C. All retention times were successively measured in triplicate; relative standard deviation of retention time was <1.4%, indicating a very good reproducibility. Therefore, only average data was used in the following calculations and figures.

The retention factor (k) was calculated using the equation $k = (t_r - t_0)/t_0$ where t_r is the retention time and t_0 is the hold-up time of TTBB. The separation factor (α) was calculated using $\alpha = k_2/k_1$. The resolution factor (R_s) was calculated using the equation $R_s = 1.18 \times (t_{r2} - t_{r1})/((w_{1/2})_1 + (w_{1/2})_2)$, where $(w_{1/2})_1$ and $(w_{1/2})_2$ are the widths at the half peak height. The symmetry factors measured by the Agilent Chemstation software.

Results

Hold-up Volume Measurements and TTBB Behavior

For end-capped CSP1, when dissolving in a hexane/IPA mixture (70/30 or 60/40, v/v), TTBB elution time decreased with increasing temperature (Figure 3). When dissolving in 100% hexane, TTBB elution time was dependent on mobile phase composition. For example, TTBB elution time did not change appreciably over the 15 - 50°C range during the hexane/IPA (70/30, v/v) mobile phase; however, elution time was slightly elongated with temperature increases for the hexane/IPA (60/40, v/v) mobile phase.

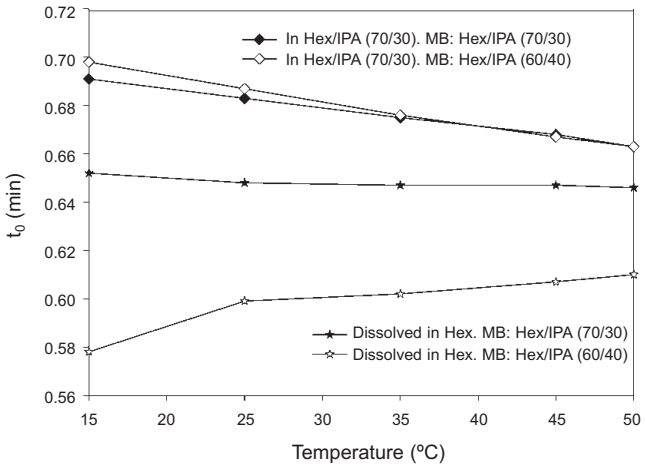

Figure 3. Variations of TTBB (0.5 mg/ml) elution time by carrier solvent and mobile phase combination (MB).

Retention Factor and Apparent TTBB Thermodynamic Parameter

The elution time of TTBB increased approximately 0.01 minute after end-capping residual silanol with hexane/IPA or hexane/MTBE mobile phase systems (Figure 4). This observation provides definitive evidence of TTBB retention on the stationary phase. This slight retention also reflects a lack of interaction between TTBB and the free silanol group. It is believed that the TTBB retention factor is obtained for the first time by using the elution time before CSP1 end-capping as the un-retained reference (Table 1). Table 1 shows that *k* decreased as the column temperature increased during the hexane/IPA mobile phase. Conversely, the *k* value increased as temperature increased during the hexane/MTBE mobile phase.

The van't Hoff plot of TTBB was not linear over the 15 - 50°C range for the hexane/IPA mobile phase (plot not shown), and thus was treated as two regions, i.e., 15 - 45°C and 45 - 50°C. The thermodynamic parameters of TTBB were then calculated for 15 - 45 °C (Table 1). The enthalpy (ΔH) and entropy (ΔS) expressed as $\Delta S^* = \Delta S/R + \ln \Phi$, where R is the gas constant, and Φ is the phase ratio were both negative, indicating an enthalpy-driven process. On the other hand, with the hexane/MTBE mobile phase, both ΔH and ΔS^* were positive, indicating an entropy-driven process.

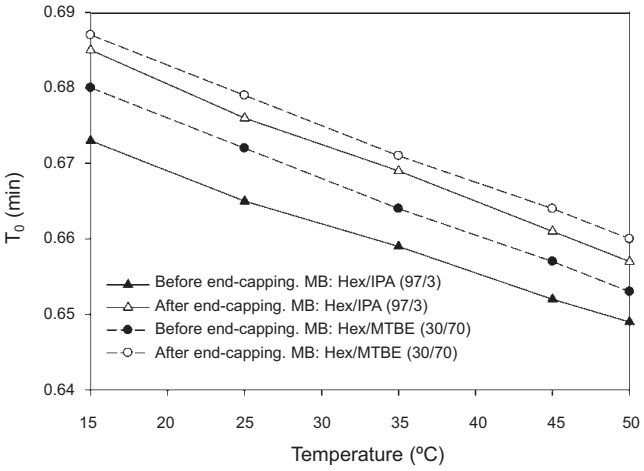

Figure 4. Effect of end-capping on TTBB elution time related to mobile phase combination (MB).

Recognize Enantioselective Mechanisms via End-Capping

Based on realizing retention behavior of TTBB, several chiral probes were then used to characterize the effects of temperature on enantioselectivity of CSP1. Chromatograms of Analyte **2** before and after end-capping are shown in Figure 5. It can be seen the pattern of peak shapes doesn't change but retention times decreased from 5.566 (the 1st peak) and 7.369 (the 2nd peak) minutes to 5.125 and 6.890 minutes, and peak widths from 0.444 (the 1st peak) and 0.642 minute (the 2nd peak) to 0.392 and 0.564 minute, respectively. The resolution increased from

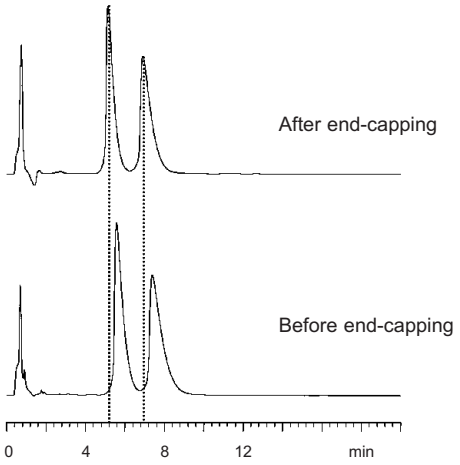

Figure 5. Comparison of Analyte 2 chromatograms before and after end-capping. Mobile phase = hexane/IPA (88/12, v/v), temperature = 50°C.

Table 1. TTBB retention factor and thermodynamic parameters. * indicates alculation of ΔH (kJ/mol) values from 15 to 45 °C.

	Hexane/IPA (97:3)				Hexane/MTBE (30:70)			
°C	*k*	ΔH (kJ/mol)	ΔS^*	r^2	*k*	ΔH (kJ/mol)	ΔS^*	r^2
15	0.0199	-0.113*	-0.027	0.998	0.0115	0.012*	0.016	0.999
25	0.0185				0.0116			
35	0.0170				0.0118			
45	0.0155				0.0119			
50	0.0138				0.0120			

1.97 to 2.18, symmetry factor from 0.326 (the 1st peak) and 0.298 (the 2nd peak) to 0.347 (the 1st peak) and 0.318 (2nd peak), respectively. It unambiguously reflects the end-capping just removed interaction of residual silanol without causing any other undesired change. With the hexane/IPA mobile phase system, retention factors of both Analytes **1** and **2** enantiomers are shorter after end-capping (Table 2; Pirkle and Readnour 1991). However, their separation factors (α) responded differently. The chiral separation of Analyte **2** was not only enhanced, as expected from the end-capping treatment, but also exhibited a linear van't Hoff plot (Figure 6). In contrast, the enantioselectivity of Analyte **1** was reduced and totally lost resolution at 50°C causing a nonlinear van't Hoff plot.

No matter whether end-capping or not, values of ΔH and ΔS^* for Analytes **1** and **2** enantiomers are all negative, signifying their retentions are enthalpy-driven on the end-capped column (Table 3). Moreover, the values of ΔH and ΔS^* for Analyte **2** enantiomers are more negative than for Analyte **1**. The in-column *in situ* process only slightly impacted the values of ΔH and ΔS^* for the 1st eluted enantiomer of Analyte **1**or **2**, while there is a relative

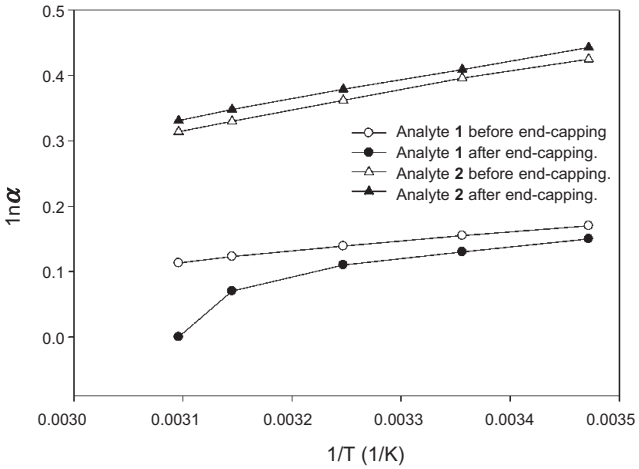

Figure 6. Influence of end-capping on chiral separations for Analytes 1 and 2. Mobile phase = hexane/IPA (88/12, v/v).

Table 2. Comparison of retention factor for Analytes 1 and 2 before and after end-capping of residual silanol. Mobile phase = hexane/IPA (88/12, v/v).

	Analyte 1				Analyte 2			
°C	k_1		k_2		k_1		k_2	
	Before	After	Before	After	Before	After	Before	After
15	3.265	3.077	3.871	3.558	21.033	18.829	32.186	29.329
25	2.593	2.416	3.027	2.762	15.533	13.558	23.073	20.413
35	2.101	1.926	2.414	2.154	11.560	10.140	16.604	14.810
45	1.722	1.581	1.947	1.702	8.766	7.829	12.189	11.086
50	1.571	1.501	1.758	1.501	7.668	6.849	10.491	9.538

Table 3. Comparison of thermodynamic parameters for Analytes 1 and 2 before and after end-capping of residual silanol. Mobile phase = hexane/IPA (88/12, v/v).

	Analyte 1						Analyte 2					
	ΔH (kJ/mol)	ΔS^*	r^2	$\Delta\Delta H$ (kJ/mol)	$\Delta\Delta S$ (J/molK)	r^2	ΔH (kJ/mol)	ΔS^*	r^2	$\Delta\Delta H$ (kJ/mol)	$\Delta\Delta S$ (J/molK)	r^2
k_1												
Before	-16.17	-5.57	1	-1.26	-0.36	0.996	-22.34	-6.28	1	-2.49	-0.61	0.998
After	-16.19	-5.65	0.996			0.925	-22.22	-6.36	1	-2.45	-0.58	0.999
k_2												
Before	-17.43	-5.93	1				-24.83	-6.90	1			
After	-19.02	-6.67	1				-24.67	-6.94	1			

large difference of ΔH between pre- and post-end-capping only for the 2nd enantiomer of the both analytes. It was interesting that reversed elution orders of Analytes **1**(+/-) and **2**(-/+) on CSP1 were observed. Because both analytes have the same type of hydrogen donor and stereo configuration (*R*(+) and *S*(-)), these differences in chromatographic behavior and thermodynamic parameter suggested their different enantiorecognition mechanisms.

Identical elution order (-/+) was observed for Analytes **3** and **4** with the hexane/MTBE mobile phase. Their chromatographic parameters listed in Table 4 shows retention times of these enantiomers are shortened after end-capping, but chiral separation factors and resolutions are improved. End-capping made values of ΔH more negative for both analytes, while the ΔS* changed from positive to negative (Table 5). These results suggested that end-capping enhanced enthalpic domination in the retention for the enantiomers.

Before end-capping, van't Hoff plots of enantioseparation for Analytes **3** and **4** showed two regions in the 15 - 50°C range (Figure 7). In the 25 - 50°C range, both $\Delta\Delta H$ and $\Delta\Delta S$ were negative for Analytes **3** and **4** (Table 5). After end-capping, their van't Hoff plots of enantioseparation in the range of 15 - 50°C showed slight nonlinear but with high correlation coefficients (r^2 = 0.998 and 0.985). Therefore, by using the linear mode to make estimation, their $\Delta\Delta H$ and $\Delta\Delta S$ values were negative and very close to values of no end-capping. As Analyte **3** is often used as a site-specific probe for testing binding sites on human serum albumin, above observations might be significant to learn its association behavior with amino acids.

Robustness Evaluation via Temperature Programs

A one-variable-at a-time (OVAT) robustness test for chiral separation was conducted by varying temperature (Injac *et al*. 2008). Fipronil, a currently used pesticide, was used as a probe in a 15-50-15°C heating-cooling temperature program with

Table 4. Comparisons of chromatographic parameters for Analytes 3 and 4 before and after the *in situ* end-capping treatment with hexane (0.1% TFA)/MTBE mobile phase (30/70, v/v).

	Analyte	k_1	k_2	α	*R*
Before	3	11.116	13.874	1.248	1.129
After	3	8.473	11.000	1.298	1.559
Before	4	9.847	11.996	1.218	1.042
After	4	7.870	9.938	1.263	1.486

Table 5. Comparison of apparent thermodynamic parameters for each enantiomer for Analytes 3 and 4 before (25 to 50°C) and after (15 to 50°C) end-capping with hexane (0.1% TFA)/MTBE mobile phase (30/70, v/v).

	Analyte 3			Analyte 4			Analyte 3			Analyte 4		
	ΔH (kJ/mol)	ΔS*	r^2	$\Delta\Delta H$ (kJ/mol)	$\Delta\Delta S$ (J/molK)	r^2	ΔH (kJ/mol)	ΔS*	r^2	$\Delta\Delta H$ (kJ/mol)	$\Delta\Delta S$ (J/molK)	r^2
k_1												
Before	-5.21	0.30	0.9986	-5.10	0.23	0.9982	-1.36	-0.33	0.9965	-1.12	-0.25	0.9932
After	-6.67	-0.56	0.9980	-6.37	-0.51	0.9997	-1.32	-0.27	0.9985	-1.01	-0.18	0.9829
k_2												
Before	-6.28	0.09	0.9961	-5.77	0.15	0.9982						
After	-7.98	-0.83	0.9981	-7.37	-0.68	0.9995						

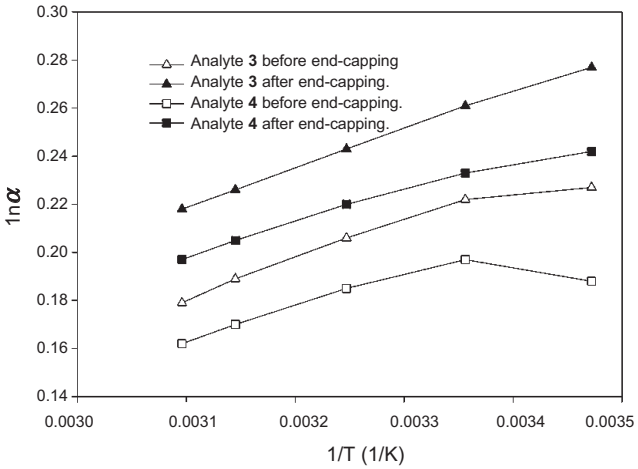

Figure 7. Influence of end-capping on chiral separations for Analytes 3 and 4. Mobile phase = hexane (0.1% trifluoroacetic acid, TFA)/MTBE (30/70, v/v).

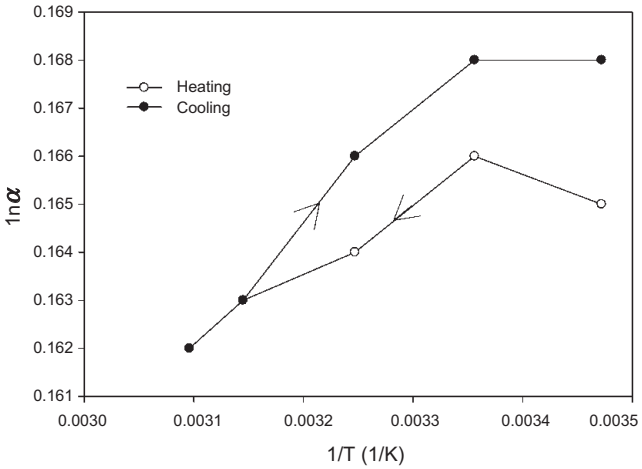

Figure 8. Response of enantioseparation for Analyte 5 in heating-cooling cyclic temperature program. Mobile phase = hexane (0.1% TFA)/MTBE (50/50, v/v). Arrows indicate direction of heating or cooling.

hexane/MTBE (50/50, v/v) mobile phase. The heating and cooling van't Hoff plots for fipronil were not linear and not superimposable over the 15 - 45°C range, but overlapped well in the 45 - 50°C range (Figure 8). The relative standard deviations for 15, 25, 35, 45 and 50°C were 0.24, 0.18, 0.12, 0, and 0.06%, respectively. The α values for 15 and 50°C in step-temperature program were exactly reproducible for Analytes 1 and 5 (data not shown), so that CSP1was considered rigid structurally (Thomas *et al*. 1994, Zhang *et al*. 2007).

Discussion

Hold-up Volume Measurement and Evaluation of TTBB Behavior

The V_0 value of CSP1 column was first measured for calculating retention factor. Although static method, i.e., pycnometry, is simple to be performed, it is hardly ever used in temperature program (Asnin *et al*. 2010). Therefore, the dynamic method employing TTBB as the marker was selected (Pirkle and Welch 1991; Lao and Gan 2007, 2008). The TTBB elution time was measured in multiple combinations of mobile phase and dissolving solvent of TTBB.

Increasing temperature can cause expansion of stainless steel column tube, while this small change of dimension is usually negligible in conventional chromatography (Gritti *et al*. 2005). Moreover, increasing temperature can also make thermal expansion of mobile phase so that the hold-up volume measured by the detector is smaller than the actual value (Lao and Gan 2007). If correction for thermal expansion of the mobile phase were made, the corrected elution times of TTBB at elevated temperatures should be even longer. The upward trend of TTBB elution time as dissolving in 100% hexane implied that there were additional factors accounting for temperature dependence of the hold-up time (Lao and Gan 2007, Asnin *et al*. 2010). In the normal phase mode, the stationary phase is expected to be covered and solvated by the mobile phase. TTBB should easily approach the surface to be retained when it was dissolved in a carrier solvent that was the same as the mobile phase (Gritti and Guiochon 2005a). In this situation, TTBB would show the "normal" phenomenon of temperature influence; that is, its elution time decreased as temperature increased. Nevertheless, as a non-polar molecule with steric hindrance, TTBB in 100% hexane was unable to compete with IPA for sorption sites on the CSP, which made TTBB "glide" cross the column at a rate relating to the mobile phase composition to give the first peak of HPLC system. Due to its minimum interactions with CSP, TTBB may be a suitable probe for evaluating solvation of stationary phase in normal phase HPLC (Lao and Gan 2008, Berthod 2009).

Retention Factor and Apparent TTBB Thermodynamic Parameter

Because the elution time of TTBB is very short, and even less than mobile phase components no reference hold-up time was available so that common method failed to measure its retention factor. In this study, by comparing elution time before and after end-

capping, TTBB retention was able to be identified, and its retention factor was calculated with the elution time on the none end-capped CSP as the reference.

The results unambiguously demonstrated TTBB retention capacity. However, the retention time was very short (<1 second for a 5-cm long column), so that it does not significantly affect the value of chromatographic parameter. Therefore, TTBB may still be considered suitable as an un-retained marker in the determination of equilibrium isotherms in nonlinear HPLC (Sajonz 2004, Gritti *et al*. 2007).

Enantioselective Mechanisms via End-capping

Since the surface of CSP1 is heterogeneous, the non-enantioselective hydrogen-bonding interaction between the residual silanol and hydroxyl group of the analytes could be one of the forces in the three-point interaction of chiral separation. Comparison of enantioselectivity before and after end-capping for Analytes **1**, **2**, **3**, and **4** suggests that the interaction model between enantiomer and CSP1 was probably different before and after end-capping (Gritti and Guiochon 2005a). Before end-capping, hydrogen-bonding or other weak interactions between the residual silanol and the enantiomer presented significant involvement in chiral recognition process. This interaction that was non-enantioselective was reduced along with temperature increase and varied with different analytes. Consequently, a nonlinear van't Hoff plot was seen for the analytes. As removing hydrogen of silanol could change the orientation of chiral selector (Pirkle and Readnour 1991), end-capping resulted in improvement or impairment of enantioselectivity depending on analytes. Reasonably, the nonlinear van't Hoff plots were still produced by some analytes in the 15 - 50°C range, probably due to interaction of remaining residual silanol after end-capping (Bidlingmeyer and Henderson 2004, Gritti and Guiochon 2006). This may also explain the reduced α value of a few analytes on end-capped CSPs (Oliveros *et al*. 1992, Hyun *et al*. 2007).

In a recent study, removal of the non-enantioselective interactions between the analytes and the residual silanol generally increased the separation factor on a doubly tethered chiral crown ether phase (Hyun *et al*. 2007). However, a few of exceptions reveal no improvement on chiral separation after end-capping. In separately bonding method, e.g., refluxing, the CSP has to undergo difficult processes in addition to being unloaded and re-packed. Although this method could have high efficiency for end-capping, unpredictable change on the CSP might be taken place. At this stage, it is really unknown what change might be invoked at elevated temperature; the *in situ* end-capping process at room temperature was an optimized choice to fulfill present study goal. The chromatographic behaviors of Analytes **1** through **4** demonstrated that this treatment is effective for identifying any *in situ* changes that may provide helpful insights on enantiorecognition mechanisms.

Evaluation via Temperature Programs

A similar temperature program was applied on several polysaccharide-based CSPs (Wang *et al*. 2008a,b). In order to directly compare results obtained with different chiral selectors and different analytes, the relative change of separation factor ($\Delta\alpha/\alpha$ (%)) in the heating and cooling temperature program was utilized. The ($\Delta\alpha/\alpha$ (%)) of 15°C was 0.34, was smaller than that for immobilized polysaccharide CSPs, e.g., Chiralpak IA and IB, although different probes were used (Wang *et al*. 2008b). Although the heating-cooling cycle temperature program shows a certain elasticity, the small α values for Analyte **5** at lower temperature, e.g. 15 or 25°C, were irreproducible. However, it is expected that nonlinear chromatography approach may offer additional information of CSP from the *in situ* change (Gritti and Guiochon 2005b).

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