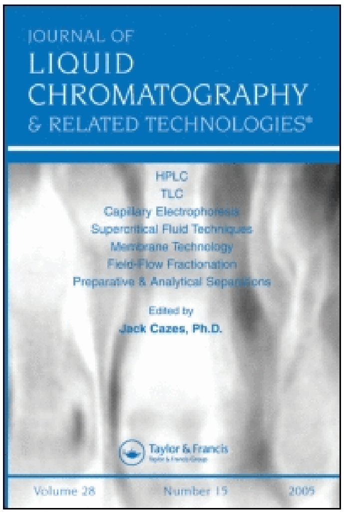

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Clausen, Andrew , Dowling, Thomas and Bicker, Gary(2002) 'DESCRIPTION OF THE RETENTION BEHAVIOR AND CHROMATOGRAPHIC MEASUREMENT OF THE CHANGE IN pKa WITH TEMPERATURE OF A DIASTEREOMERIC PAIR OF ISOLEUCINE DERIVATIVES', Journal of Liquid Chromatography & Related Technologies, 25: 5, 705 — 715

To link to this Article: DOI: 10.1081/JLC-120003029

URL: http://dx.doi.org/10.1081/JLC-120003029

J. LIQ. CHROM. & REL. TECHNOL., 25(5), 705–715 (2002)

DESCRIPTION OF THE RETENTION BEHAVIOR AND CHROMATOGRAPHIC MEASUREMENT OF THE CHANGE IN pKa WITH TEMPERATURE OF A DIASTEREOMERIC PAIR OF ISOLEUCINE DERIVATIVES

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ABSTRACT

The retention of a diastereomeric pair of peptide analogs was investigated as a function of temperature using reversed-phase HPLC. The retention of these compounds showed anomalous behavior with retention increasing with increasing temperature. The change in the compounds' pK_a as temperature was varied was measured chromatographically and decreases with increasing temperature. The change in degree of ionization as a function of temperature was used to explain this anomalous retention behavior.

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INTRODUCTION

The optimization of selectivity within a reasonable retention window ($1 \leq k \leq 20$) is often the goal during HPLC method development. Many factors can be varied to achieve desired selectivity. The most popular and often used of these variables are solvent strength, solvent type, pH, and column type (1,2). Temperature has also proven to be a powerful and convenient way to affect selectivity, especially when the separation involves ionogenic compounds such as peptides (3).

The change in retention in reversed-phase LC (RPLC) as temperature is varied, can typically be described by the van't Hoff relationship: (4)

$$\ln k' = A + \frac{B}{T} \tag{1}$$

where k' is the retention factor, T is the absolute temperature, A is a function of the phase ratio and entropy of retention, and B is proportional to the enthalpy of retention. In general, retention of solutes will decrease with increasing temperature following this linear relationship.(4–7) Deviations from linearity can occur due to changes in stationary phase conformation (8,9), retention due to more than one mechanism such as silanophilic interactions (3), and cases where there are secondary equilibria such as acid-base equilibria.(2)

Deviations from linearity in retention and temperature dependence due to acid-base equilibria, can be attributed to the perturbation of the $\mathrm{pK_a}$ of a solute induced by changing temperature, and are some of the reasons why varying temperature can provide useful changes in selectivity. The theory of retention of monoprotic acids and bases as a function of pH is available.(10,11) For monoprotic bases, retention of the ionized or protonated form is less than that of the more hydrophobic neutral or deprotonated form; therefore, the capacity factor of a base at a given pH depends on the fraction of the compound that exists in the protonated or ionized state (F^+) given by:

$$F^+ = \frac{1}{\left\{1 + \left(\frac{K_a}{[H^+]}\right)\right\}} \tag{2}$$

where the fraction ionized is a function of the dissociation constant K_a and the mobile phase pH. The relationship between capacity factor and pH is then described by the equation:

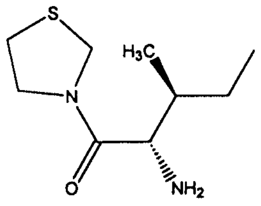
$$k' = k^{\mathrm{o}}(1 - F^+) + k^+ F^+ \tag{3}$$

where k^{o} and k^+ refer to capacity factors for the neutral form of the base and the protonated form of the base respectively.

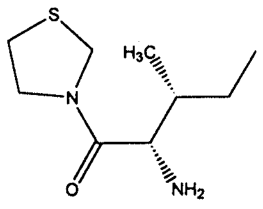
While the pH of a buffer is not significantly affected by temperature, the $\mathrm{pK_a}$ of bases in water decreases as the absolute temperature increases (12) following the approximate relationship:

$$-\frac{d(\mathrm{pK_a})}{dT} = \frac{(\mathrm{pK_a} - 0.9)}{T} \tag{4}$$

This equation agrees well with experimental data over the range of 15–35°C in water. It implies that the stronger a base the greater the change in $\mathrm{pK_a}$ with temperature. The change in $\mathrm{pK_a}$ of a base adds a layer of complexity when describing the retention-temperature dependence especially if the pH of the mobile phase is close to the $\mathrm{pK_a}$ of the compound and the temperature range studied is large. The aim of this study is not to describe a detailed theoretical analysis but rather to present the retention behavior of two peptide analogs, a diastereomeric pair of bases 2S,3S-isoleucyl thiazolidide (*threo*) and 2S,3R-isoleucyl thiazolidide (*allo*) shown below and offer a brief rationalization for the retention-temperature dependence that was observed.



Threo



Allo

EXPERIMENTAL

Instrumentation

The chromatographic system used consisted of an HP 1100 vacuum degrasser, quaternary pump, autosampler, thermostated column compartment, and variable wave length detector (Wilmington, DE, USA). Chromatography data was collected and processed by TurboChrom 6.1 (Perkin Elmer, Cupertino, CA, USA). The pH meter used for these studies was a Brinkmann Model 713 (Lauda-Konigshofen, Germany), and the automatic titrator, also from Brinkmann, was a 716 DMS Titrino.

Materials

HPLC-grade acetonitrile was from EM Science (Gibbstown, NJ, USA). Potassium dihydrogen phosphate and di-potassium hydrogen phosphate used for buffer preparation were purchased from Fluka (Milwaukee, WI, USA). Calibration solutions buffered at pH four, seven, and ten were from Fisher Scientific (Fair Lawn, NJ, USA). The 2S,3S-isoleucyl thiazolidide hemifumarate salt was purchased from Heumann Pharma (Nuremberg, Germany). The 2S,3R-isoleucyl thiazolidide hemifurmarate diastereomer was provided by the Process Research department of Merck Research Laboratories (Rahway, NJ, USA).

Measurement of pK_as

The pK_a of each diastereomer in water was measured in triplicate. Approximately 200 mg of the compound was dissolved in water and titrated against standardized 0.1 N hydrochloric acid. Progression of the titration was followed potentiometrically after addition of each aliquot of acid solution.

Chromatographic Conditions

The column used in this study was an ACE C18 (5 µm, 250 × 4.6 mm, Advanced Chromatography Technologies, distributed by MAC-MOD Analytical, Chadds Ford, PA, USA). The aqueous mobile phases were prepared by weighing out the appropriate amount of potassium dihydrogen phosphate and di-potassium hydrogen phosphate in ratios that would give the desired concentration and pH. The organic mobile phase (denoted B) was prepared by first mixing seventy volume percent acetonitrile with thirty volume percent water, and then by adding the same amounts of potassium dihydrogen phosphate and di-potassium hydrogen phosphate to this solution that were used to prepare the completely aqueous mobile phase (denoted A). The masses of potassium dihydrogen phosphate and di-potassium hydrogen phosphate for pH control for each mobile phase preparation can be found in Table 1.

The pH of the completely aqueous mobile phases (denoted pH^{aq}) were measured by first calibrating the pH meter against buffers at pH four and ten and checking this calibration against a pH seven buffer, after which the pH^{aq} of the aqueous mobile phase was measured. The apparent or effective pH^{e} of the HPLC mobile phase composition used in these studies was measured in the same manner as that of the completely aqueous mobile phase. While this is not a rigorous method for determining solution pH in the presence of organic modifier, it has been recommended and shown to be a useful determination (13).

Table 1. Mobile Phase Conditions

Mobile Phase #	KH_2PO_4 (g/L)	K_2HPO_4 (g/L)	$[PO_4^{-3}]$ (mM)	$[K^+]$ (mM)	Mobile Phase Ratio A : B (% ACN)	pH^{aq}
1	0.700	–	5.1	5.1	68 : 32 (22.4)	4.9
2	0.612	0.087	5.0	5.5	68 : 32 (22.4)	6.1
3	0.408	0.307	4.8	6.5	68 : 32 (22.4)	6.8
4	0.095	0.749	5.0	9.3	68 : 32 (22.4)	7.8
5	–	0.870	5.0	10.0	68 : 32 (22.4)	9.1

The measured pH values, as well as the mobile phase compositions for each experiment, also appear in Table 1.

RESULTS

Effect of Temperature on Retention of Basic Diastereomers

Figure 1a (pH^{aq} 6.1), Figure 1b (pH^{aq} 6.8), and Figure 1c (pH^{aq} 7.8) are van't Hoff plots showing the effect of temperature on the retention and separation of the diastereomers over the temperature range 0–60°C. The pK_a of the diastereomers in water was measured at 7.5 at 20°C. The pH^{aq} of the three mobile phases brackets the pK_a of the diastereomers within 1.7 pH units, or well within the region where there will be a fraction of the compound in both the ionized and neutral forms.

As is frequently seen (14), over a narrow temperature range of 30 degrees these plots exhibit some linearity, but over the entire range studied there is significant curvature to the plots. In addition, all exhibit a net negative slope as retention increased with increasing temperature. For ionogenic compounds such as these diastereomers, and at mobile phase pHs near to the compounds' pK_a, the degree of ionization and the change in ionization with temperature is certainly a consideration.

Measurement of pK_as as a Function of Temperature

The pK_a of bases decreases as the absolute temperature increases (12). As the temperature was varied for the studies described above, the pK_a of the diastereomers and, thus, the degree of ionization was changing.

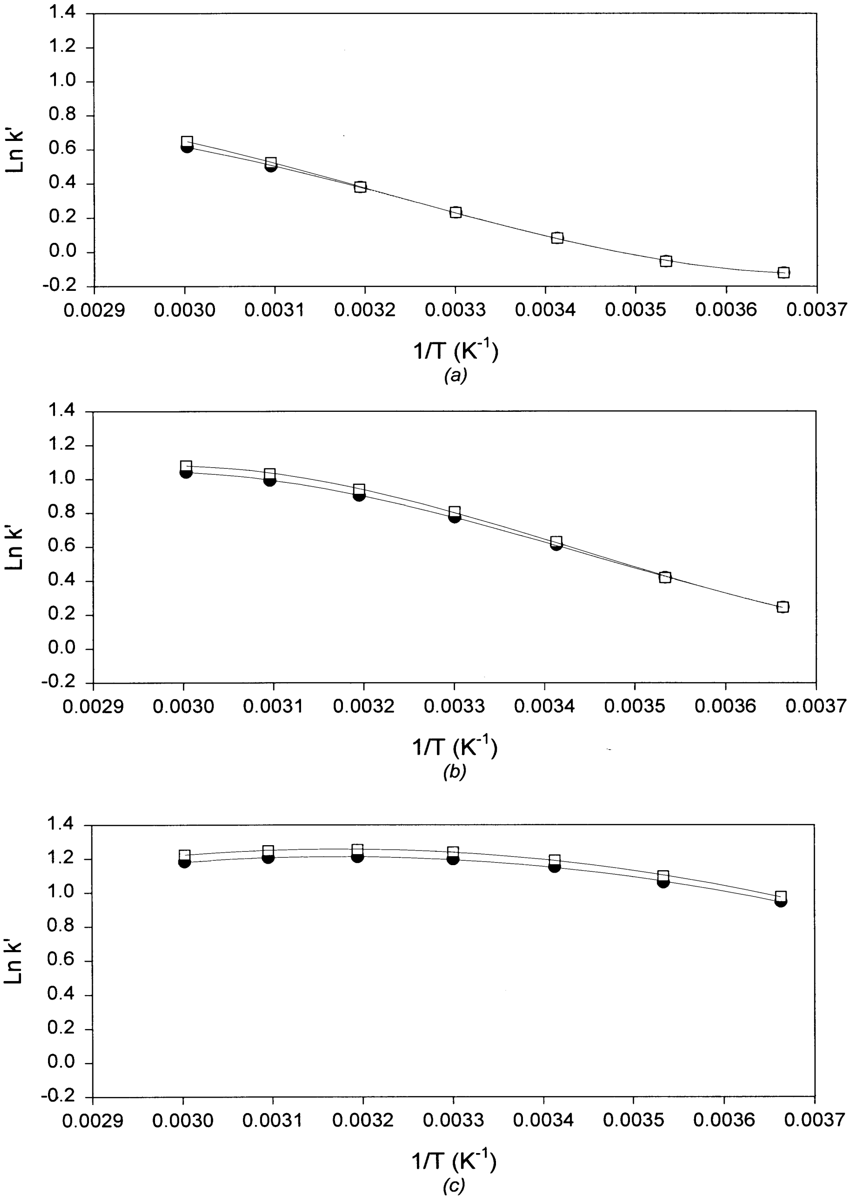


Figure 1. van't Hoff plots for the *threo*- (●) and *allo*- (☐) diastereomers at pH^{aq} (a) 6.1, (b) 6.8 and (c) 7.8. Chromatographic conditions: mobile phase #2, #3, #4 see Table 1; flow rate, 1.0 ml/min; detection, UV 215 nm; sample, 5 μl injection of a 0.4 mg/mL *threo*- and 0.3 mg/mL *allo*- solution.

In Figure 2a (*allo*) and 2b (*threo*) the capacity factors at temperatures from 0 to 60°C are plotted against the pH of the aqueous mobile phase, which ranged between 4.9 and 9.1. These data were then fit to the combined form of equations 2 and 3 by setting k^{o} as the capacity factor at pH^{aq} 9.1 and k^{+} to the capacity factor measured at pH^{aq} 4.9 for each temperature, respectively. The correlation

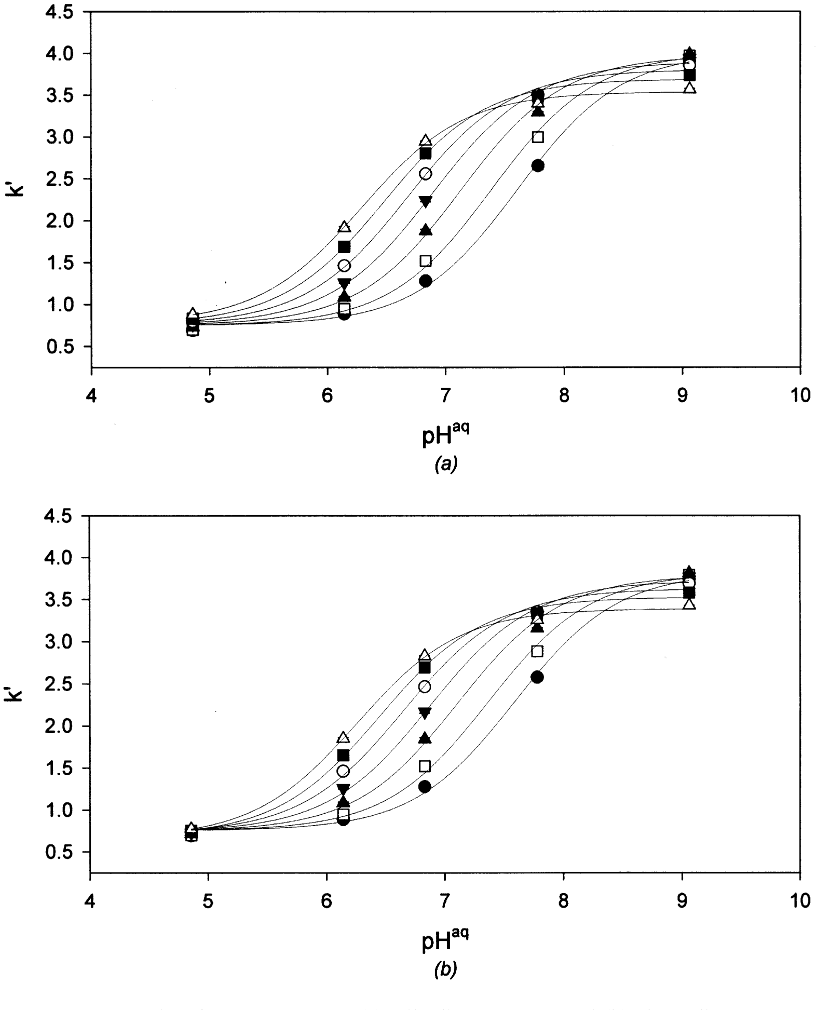


Figure 2. Retention factor versus pH (a) *allo* diastereomer and (b) *threo* diastereomer at 0 (•), 10 (□), 20 (▲), 30 (▼), 40 (○), 50 (■), and 60 (△) C. Chromatographic conditions: mobile phase #1, #2, #3, #4, #5; other conditions were the same as Figure 1.

coefficient for these fits were all greater than 0.997. Fitting the data to this equation allowed for the estimation of pK_a for the diastereomers under these mobile phase conditions and column temperatures.

The pK_as determined in the above manner for this solvent system are plotted versus temperature in Figure 3. The decrease in pK_a is linear with temperature over the range studied. The change in pK_a per degree for both diastereomers is the same at 0.022, which agrees well with the value 0.023 calculated using equation 4.

Using the pK_a determined at each temperature, the fraction ionized for mobile phases of pH^{aq} of 6.1, 6.8, and 7.8 was calculated and plotted versus

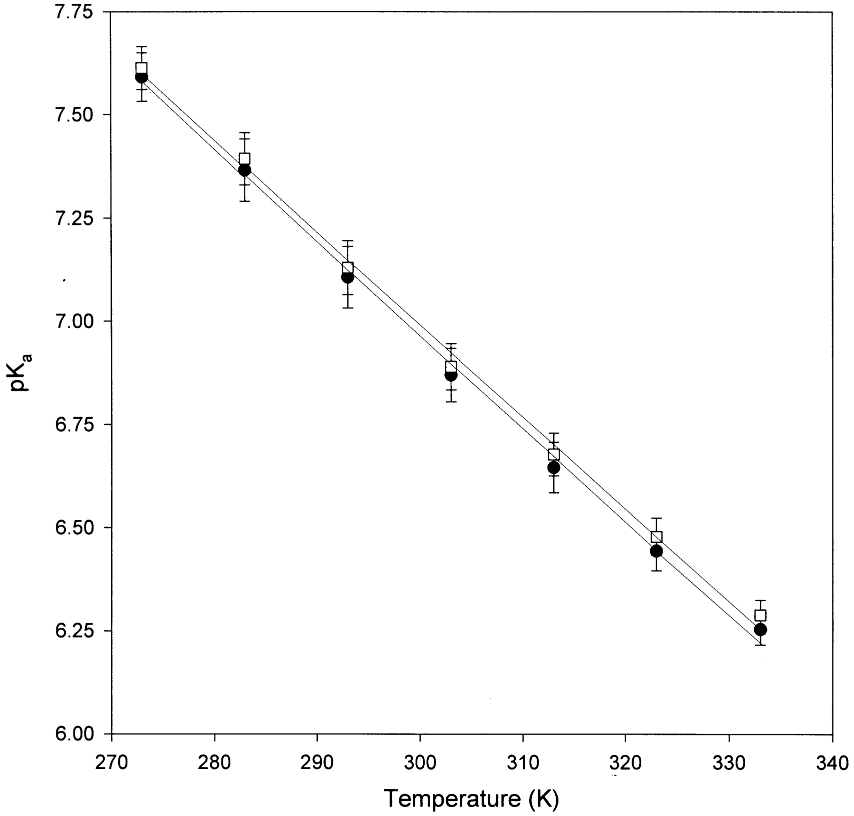


Figure 3. Temperature versus chromatographically determined pK_a of the *threo-* (●) and *allo-* (□) diastereomer with linear regression (—) results; error bars are the standard error of the regression coefficient.

temperature in Figure 4. This plot illustrates the change in the degree of ionization that occurs over this temperature range. We note, that under these mobile phase conditions, separation was achieved only when the fraction ionized was less than 0.7.

The change in the fraction ionized is the source of the deviation from linearity and negative slope observed for the van't Hoff plots. Increasing the temperature reduced the fraction, which was protonated or increased the fraction that was not charged. The more hydrophobic neutral form of the diastereomers was more retained, allowing for a net increase of solute retention with increasing temperature. Increased retention, due to increasing the fraction of the neutral diastereomer with increasing temperature, is still coupled to the effect increasing column temperature has on the strength of hydrophobic interactions that drive

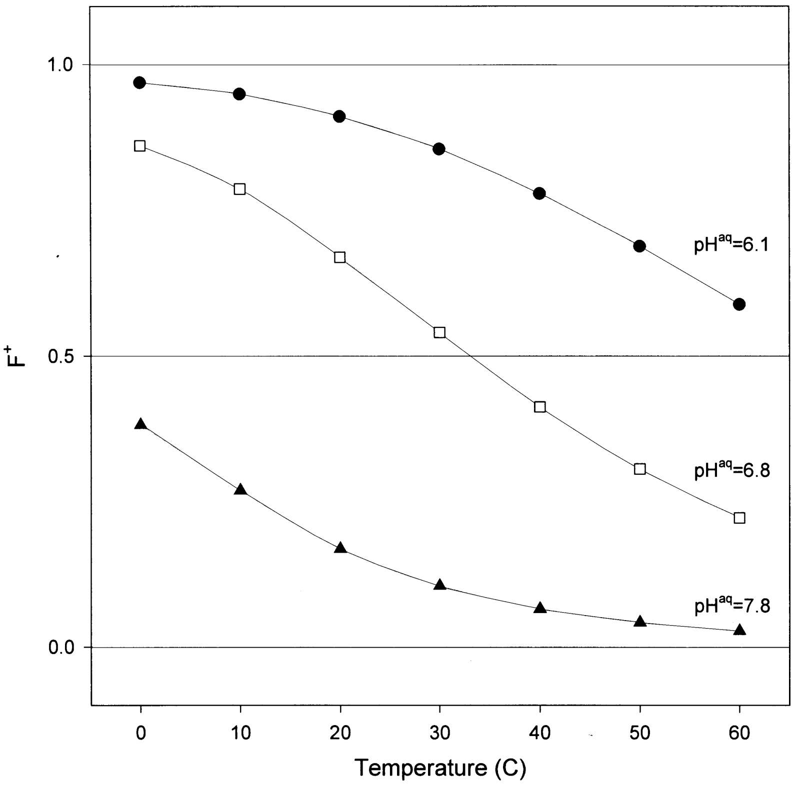


Figure 4. Fraction of the diastereomers protonated at each aqueous mobile phase pH versus temperature.

retention. The strength of hydrophobic interactions decreases with temperature, as described by the van't Hoff equation. Examination of Figure 1c shows an example of this. Retention increases to a maximum of retention near 40°C due to increased fraction of the neutral form. This is followed by a slight decrease in retention. Above 40°C, while the pK_a is still decreasing and the fraction of the neutral form of the compound is still increasing at a slower rate, the decrease in the strength of the hydrophobic interactions is evident from the decrease in retention as temperature is increased.

CONCLUSIONS

Column temperature can be a useful tool when attempting to optimize selectivity during method development, especially for ionogenic compounds. For this case of the reversed-phase separation of these *allo*- and *threo*- isoleucine derivatives, changing column temperature from 0 to 60°C allowed for the separation of these compounds. This change in temperature caused a 1.4 unit shift in the compounds' pK_a which resulted in a significant shift in the relative amounts of protonated and neutral forms of the bases, allowing for greater hydrophobic interactions and greater selectivity.

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Received October 1, 2001
Accepted October 24, 2001
Manuscript 5665