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Journal of Chromatography A, 756 (1996) 51–62

JOURNAL OF CHROMATOGRAPHY A

Combined use of temperature and solvent strength in reversed-phase gradient elution
III. Selectivity for ionizable samples as a function of sample type and pH

P.L. Zhu[1,a], J.W. Dolan[a], L.R. Snyder[a,*], D.W. Hill[b], L. Van Heukelem[c], T.J. Waeghe[d]

[a]*LC Resources Inc., Walnut Creek, CA 94596, USA*

[b]*Microchemistry Laboratory, University of Connecticut, Storrs, CT 06269, USA*

[c]*Horn Point Environmental Laboratory, Center for Environmental and Estuarine Studies, University of Maryland, PO Box 775, Cambridge, MD 21613, USA*

[d]*Du Pont Agricultural Products, Experimental Station, Wilmington, DE 19880, USA*

Received 29 April 1996; revised 17 July 1996; accepted 31 July 1996

Abstract

The ability of temperature and gradient steepness to change band spacing has been investigated for several ionizable samples that include 8 substituted benzoic acids, 9 substituted anilines, 22 basic drugs, 9 structurally-related herbicide impurities, 7 chlorophylls and 72 peptides and proteins. Mobile phase pH was also varied to determine the effect of sample ionization on temperature and gradient-steepness selectivity.

Keywords: Selectivity; Column temperature; Gradient steepness; Gradient elution; Benzoic acids; Anilines; Chlorophylls; Peptides; Proteins; Pesticides; Basic drugs

1. Introduction

Changes in band spacing with temperature are not expected for samples whose components are formed from identical, repeating units (homologs, benzologs, oligomers). For compounds of this type, "regular" temperature behavior is the rule (Part II, [1]). Samples containing other kinds of compounds will be less "regular", as a result of various circumstances summarized in Table 1. The contributions to temperature selectivity of Table 1 can be summarized by saying that "regular" samples are retained by a single retention process that is unaffected by other processes such as solute isomerization or ionization, ion-pair formation, competitive retention of mobile phase additives such as amines or ion-pair reagents, changes in conformation of solute or stationary phase, etc. This rarely will be exactly the case.

Temperature-selectivity effects 2–4 of Table 1 involve ionizable solutes and generally will be of major importance; i.e. acidic or basic samples should

* Corresponding author.

[1] Permanent address: Department of Chemistry, Lanzhou University, Gansu province, China.

0021-9673/96/$15.00
PII S0021-9673(96)00723-6

Table 1
Situations favoring temperature selectivity ("irregular" behavior)

1	When a solute molecule can interconvert rapidly to an isomer or conformer; see the discussion of [2].
2	When an acidic or basic solute is partially ionized, so that the molecule exists in both ionized and un-ionized forms; this case is similar to that of solute isomerization (1).
3	When ion-pairing is used, solute retention can occur by both hydrophobic and ion-exchange interaction; also, retention depends on the amount of ion-pair reagent taken up by the stationary phase [3].
4	When a mobile phase additive is used (e.g. an amine modifier) which is retained and affects the retention of basic sample solutes by blocking silanols.
5	When the relative retention of two solutes is sensitive to changes in the conformation of the stationary phase as temperature is varied [4].
6	When the relative size or shape of two molecules is different, leading to differences in their entropies of solution [5].
7	When two molecules have different functional groups, and the temperature dependence for the retention of these groups is not the same; this will likely be the case (to some degree) for most non-homologous molecules, since different functional groups interact differently with the mobile and stationary phases (varying contributions from dispersion, dipole induction and orientation, and hydrogen bonding interactions [6–8]).

be more "irregular". Furthermore, effects 2–4 should vary with mobile phase pH and tend to be larger when the solute is half-ionized (when pH≈pK_a for the sample). The largest temperature-selectivity effects are expected for ion-pair separations of acids or bases, and this is confirmed by practical experience [9,10].

This paper examines temperature (T) and gradient-steepness (b) selectivities for different ionic samples and different separation conditions, including mobile phase pH. One goal of this study is to determine whether these selectivity effects are generally significant and worth exploiting during HPLC method development. A second goal is to determine if T- and b-selectivities are correlated or act independently. If these two selectivities act independently, it is worthwhile to vary temperature and gradient steepness simultaneously during method development. A third goal is to see how T- and b-selectivities depend on the molecular structure of sample compounds. The use of temperature and/or gradient steepness as a means of varying band spacing may not be useful for some (pre-identifiable) samples. A final goal is to determine the effect of other separation conditions on T- and b-selectivity, which would allow the selection of preferred initial conditions (e.g. solvents, pH, column type, etc.) for different samples, using the present method development approach (Fig. 2 of Part I [11]). A similar investigation for neutral samples (with similar goals) is reported in Part IV [12].

2. Experimental

Except for the chlorophyll sample, all retention time values discussed in this paper are from either Part I [11] or [9]. Experimental conditions for the chlorophyll sample (laboratory G) are given in Part IV [12].

2.1. Experimental reproducibility

Retention data to be used for selectivity measurements should be as precise as possible (e.g. ±1–2%). If solutes are injected individually over an extended period, changes in the column combined with small errors in the formulation of new mobile phase can lead to unacceptable variations in retention time and derived values of α. It is also important, especially for gradient elution measurements as in the present study, to ensure that the column is equilibrated with the A-solvent prior to sample injection and start of the gradient.

In the present study and Part IV [12], various expedients were used to minimize experimental error and to test for the reliability of the data reported here. In most cases, several solutes were separated simultaneously, rather than injecting individual solutes. Individual samples were studied over a short time period, using the same column. Replicate injections were carried out to confirm that there was no change in the column from start to finish. When

data gave unexpected correlations, these data were checked by rerunning the sample. Computer simulations and other chromatographic relationships were also used to test the internal consistency of the data reported here. On the basis of these and other practices, it is believed that values of α from the present studies are reproducible within $\pm 1\%$.

3. Results and discussion

The gradient separations of several ionic samples were studied as a function of temperature and gradient steepness, and, in some cases, mobile phase pH. These investigations are discussed below for each sample.

3.1. Substituted benzoic acid sample

This eight-component sample (Table 1 of Ref. [12], solutes 1–8) was separated at temperatures of 24.6–69.7°C, with acetonitrile–buffer mobile phases (phosphate/citrate buffers; gradient times of 15 and 45 min; pH=2.6, 3.2, 3.7 and 4.3). The pK_a values of these solutes have been determined in methanol–water [13] mobile phases (similar %-organic) and are summarized in Table 2.

3.1.1. Temperature selectivity

Differences in retention time $\Delta t_R=(t_R)_a-(t_R)_b$ were determined at each pH for temperatures of $T_a=24.6$°C and $T_b=69.7$°C, with both a 15 and a 45 min gradient. These values of Δt_R are plotted vs. t_R (32.1°C, 45-min run) in Fig. 1 at each pH in order to obtain deviations $\delta(\Delta t_R)$, which measure the degree of "irregular" temperature behavior. Values of $\delta(t_R)$ are equal to the standard error of Y (i.e. Δt_R) in Fig. 1. Average values of $\Delta\log\alpha^*(T)$ were calculated from $\delta(t_R)$ for each pH as described in Part II (Eqs. 13 and 14 [1]). Values of $\Delta\log\alpha^*(T)$ represent average changes in α for a given sample and set of conditions for a (calculated) 60°C change in T, corrected for any trend in α with retention time. Values of $\Delta\log\alpha^*(T)$ are thus a measure of the ability of a change in T to effect the separation of a previously overlapped band-pair. Values of $\Delta\log\alpha^*(b)$ are a similar measure of the ability of a change in %B or gradient steepness b to effect the separation of an overlapped band-pair.

A similar calculation (as in Fig. 1) for the 15-min gradients was also carried out in order to obtain a second value of $\Delta\log\alpha^*(T)$ for each sample. The average values of $\Delta\log\alpha^*(T)$ (for gradient times 15 and 45 min) are summarized in Table 3 as a function of pH.

The value of $\Delta\log\alpha^*(T)$ corresponding to a maximum change in temperature (60°C) is significant (>0.02) for each pH; average $\Delta\log\alpha^*(T)$= 0.07–0.11, corresponding to changes in α of 17–29%. The value of $\Delta\log\alpha^*(T)$ tends to increase with higher pH, corresponding to increased ionization of the sample. When pH=pK_a, maximum changes in solute ionization can be expected for a change in any variable (T in the present case) that affects either pH

Table 2
Values of pK_a for benzoic acid (BA) and aniline (An) solutes under reversed-phase conditions (laboratory A)

Compound		pK_a	Compound		pK_a
1	2-Nitro BA	2.7	9	4-Methoxy An	4.2
2	Phthalic acid	3.2	10	3-Methyl An	4.0
3	2-Fluoro BA	3.6	11	2-Chloro An	2.1
4	3-Cyano BA	3.5	12	4-Chloro An	3.3
5	2-Chloro BA	3.2	13	3-Chloro An	2.9
6	3-Nitro BA	3.4	14	3,5-Dimethyl An	4.0
7	3-Fluoro BA	3.8	15	*N*-Ethyl An	4.3
8	2,6-Dimethyl BA	3.5	16	3,4-Dichloro An	2.3
			17	3,5-Dichloro An	2.0
	Average	3.3		Average	3.2
				Average (excluding $pK_a<2.6$)	3.8

Average values from Ref. [13] for methanol–water as solvent.

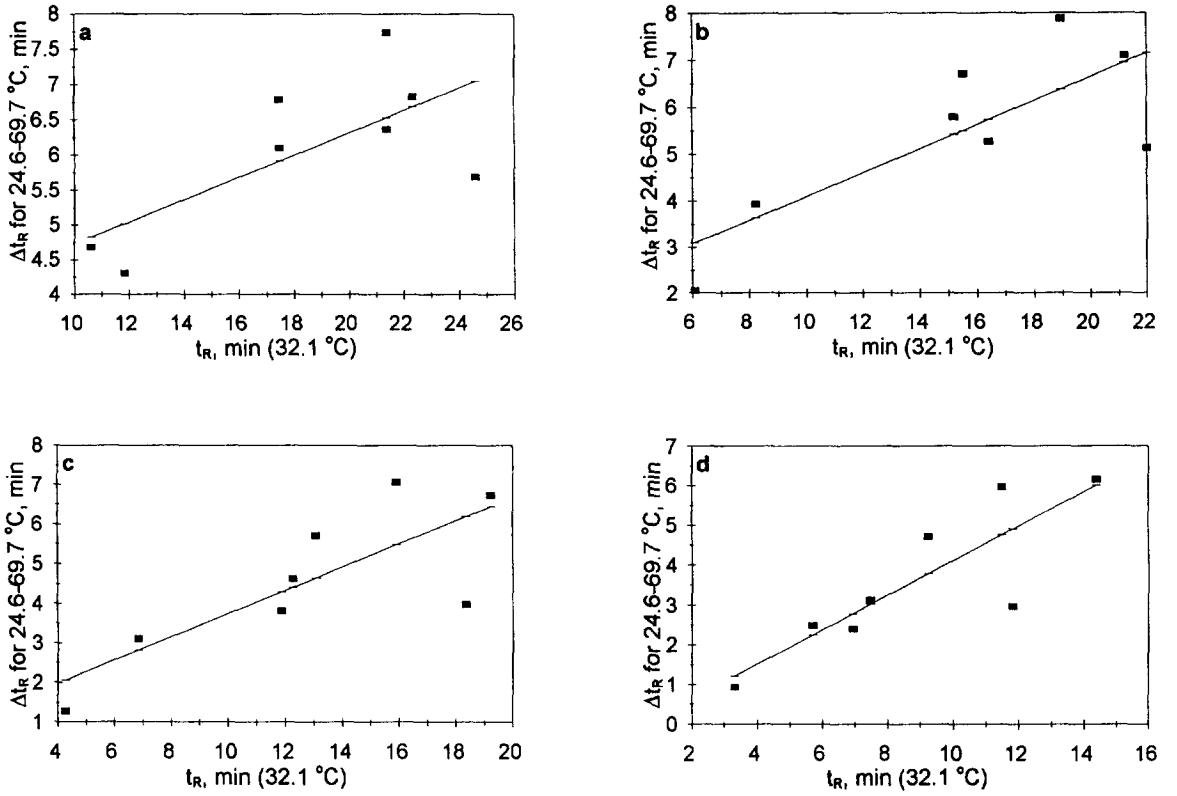


Fig. 1. Correlation of values of Δt_R vs. retention time for benzoic acid sample at 32.1°C (45-min gradient). (a) pH 2.6; (b) pH 3.2; (c) pH 3.7; (d) pH 4.3. See Section 3.1.1 for details.

or pK_a. This in turn might be expected to lead to greater selectivity for that variable when $pH=pK_a$. However, this does not occur for the benzoic acid sample; $\Delta\log \alpha^*(T)$ is constant within experimental error for $3.2<pH<4.2$, and the average pK_a for the sample is estimated to be 3.3 (Table 2).

3.1.2. Gradient-steepness selectivity

Values of S at 32.1 and 50.9°C were obtained for each sample component at different pH-values and plotted vs. retention time at that pH (45-min gradients). Fig. 2 shows resulting plots of S vs. t_R at each pH (T=32.1°C) for the benzoic acid sample. The greater the scatter in these plots, the greater is b-selectivity for that sample. Values of δS were obtained from these and other plots (standard error in Y, similar to the determination of $\delta[\Delta t_R]$ values from the plots of Fig. 1) and used to calculate values of $\Delta\log \alpha^*(b)$ (Eq. 7a, Part II). The latter values are shown in Table 4 and range from 0.21 at pH 2.6 to 0.16 at pH 4.3; i.e. values of $\Delta\log \alpha^*(b)$ tend to be larger at low pH, but not by much.

Fig. 3 shows corresponding plots of S vs. Δt_R for each pH (benzoic acid sample). Since it was shown in Part II ([1]) that S does not vary much with temperature, average values of S for each solute were used (temperatures of 24.6, 32.1, 50.9 and 69.7°C). The average of four values is less subject to error, compared to individual S-values. The data of Fig. 3 are scattered about the least-squares fit (solid line), implying that T- and b-selectivities are uncorrelated and can therefore be used together to control band spacing. Values of the correlation coefficient r^2 from each of these plots (Table 4) vary from 0.01 to 0.13, suggesting little correlation of T- and b-selectivities.

Table 3
Summary of temperature-selectivity as a function of sample type and pH (ionizable samples)

Sample	n[a]	T[b] (°C)	t_G[c] (min)	Average $\Delta\log\alpha^*(T)$		
				Low t_G	High t_G	Average[d]
Benzoic acids[e]						
pH 2.6	8	24.6, 69.7	15, 45	0.06	0.07	0.07
pH 3.2				0.11	0.09	0.10
pH 3.7				0.13	0.10	0.11
pH 4.3				0.14	0.08	0.11
Anilines[e]						
pH 2.6	9	25.5, 69.7	1, 3 %B/min[f]	0.09	0.06	0.07
pH 3.6				0.20	0.10	0.15
pH 4.6				0.13	0.15	0.14
pH 5.6				0.08	0.09	0.09
Basic drugs[g]	22	30, 66.3	20, 60	0.14	0.09	0.11
Herbicide sample[h]	9	39.9, 57.3	10, 30	0.21	0.16	0.14
Chlorophylls[i]	7	40, 60	25, 75	0.27	0.16	0.21
rhGH peptides[j]	19	20, 60	30, 120	0.34	0.36	0.35
rt-PA peptides[j]	38	40, 60	60, 120	0.33	0.31	0.32
Cereal proteins[j]	15	50, 70	60, 120	0.36	0.40	0.38
Average				0.20	0.17	0.18
Average dev.[k]						0.02

[a] Number of compounds in sample.
[b] Two temperatures used to calculate Δt_R.
[c] Low and high gradient times; e.g. 15 min is the value of "low t_G" and 45 min is the value of "high t_G".
[d] Average of low and high t_G values, arbitrarily rounded to favor the high t_G value.
[e] Data from laboratory A.
[f] Values of t_G and $\Delta\phi$ varied for different values of pH (see Appendix I of Ref. [11]); gradient steepness (%B/min) was either 1 or 3 %B/min in the two runs with varying t_G.
[g] Data from laboratory B.
[h] Data from laboratory G.
[i] Data from laboratory F.
[j] Data of Refs. [9,17].
[k] Agreement of low and high t_G values with average of two values (overall average for all samples).

3.1.3. Isocratic data, methanol–water mobile phases

Retention data have been reported [14] for a similar substituted benzoic acid sample (same compounds plus an impurity, $n=9$) separated isocratically as a function of % MeOH–water, temperature and pH. For pH 2.6–3.2, average values can be derived of $\Delta\log\alpha^*(T)=0.06$ and $\Delta\log\alpha^*(b)=0.14$. These values of $\Delta\log\alpha^*$ can be compared to corresponding average values in Table 3 (0.085 and 0.23, respectively), recognizing the differences in both sample and mobile phase (MeOH vs. ACN). The nine-component sample of [14] could be separated with $R_s>2$ by optimizing temperature and %-MeOH simultaneously [15]. The latter two variables were more effective than changes in pH or buffer concentration in maximizing resolution for this sample (for a starting pH 2.9, but not necessarily for other pH-values).

3.2. Substituted aniline sample

This nine-component sample (Table 1 of Ref. [11]) was separated at temperatures of 25.5–69.7°C, with acetonitrile–buffer mobile phases whose pH was varied from 2.6 to 5.6 (gradient steepness=1 or

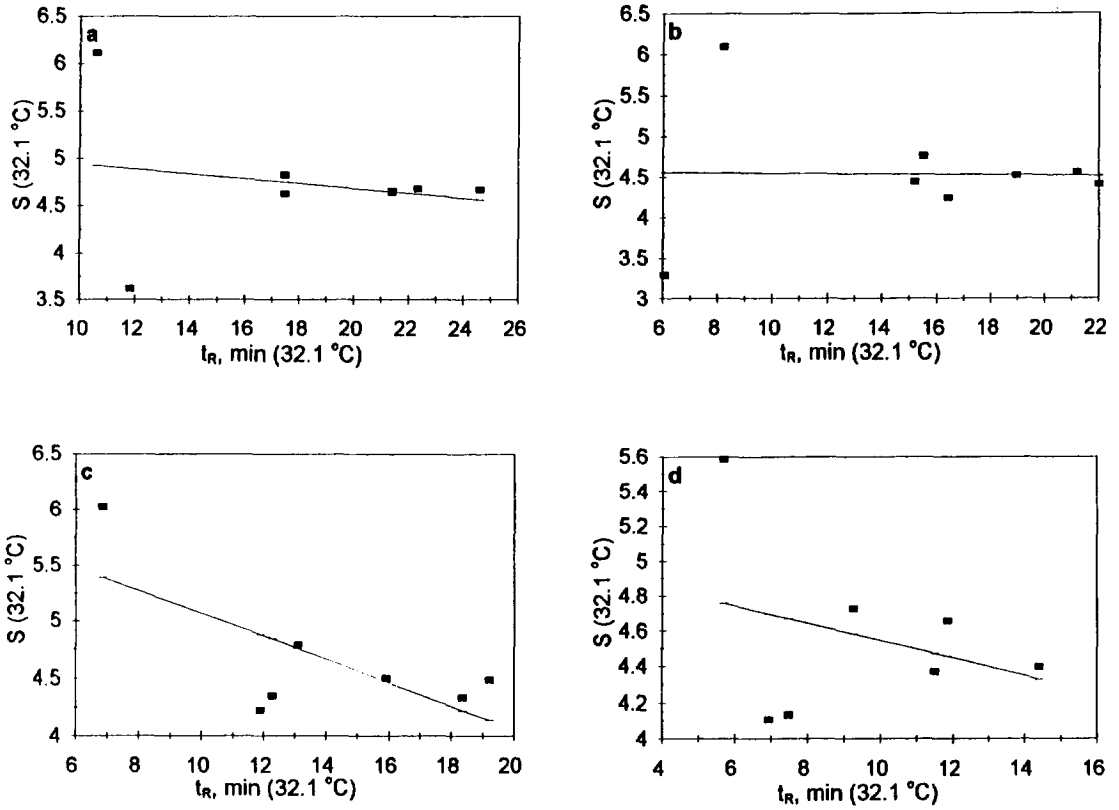


Fig. 2. Correlation of values of S vs. retention time for benzoic acid sample at 32.1°C. (a) pH 2.6; (b) pH 3.2; (c) pH 3.7; (d) pH 4.3. See Section 3.1.2 for details.

3%/min; same as for the benzoic acid sample). The pK_a values of these solutes have been determined in methanol–water [13] mobile phases (similar %-organic); see Table 2. Three of these compounds (11, 17, 18) have $pK_a<2.6$ and are therefore largely un-ionized in the pH range studied (2.6–5.6). The remaining six anilines have an average pK_a value of 3.8. Plots of S and Δt_R vs. t_R for these compounds at each pH gave correlations similar to those of Fig. 1 and Fig. 2.

3.2.1. Temperature selectivity

Average values of $\Delta\log\alpha^*(T)$ for the aniline sample were determined for each pH (Table 3), and are seen to be similar (0.07–0.15) to values for the benzoic acids (0.07–0.11). Temperature selectivity for the anilines reaches a maximum value ($\log\alpha^*(T)=0.15$) in the vicinity of pH 4.1, which is close to the average pK_a value of the sample ($pK_a=3.8$).

3.2.2. Gradient-steepness selectivity

Table 4 lists values of $\Delta\log\alpha^*(b)$ for the anilines at each pH. With the exception of the pH 2.6 run, the magnitude of $\Delta\log\alpha^*(b)$ (0.13–0.20) is similar to values for the benzoic acids (0.13–0.25). Isocratic data for methanol as B-solvent were reported previously [13] for the same sample plus an added solute (3-cyanoaniline). These data allow similar calculations of $\Delta\log\alpha^*(b)$ as a function of pH: pH 2.0, 0.22; pH 3.0, 0.27; pH 4.5, 0.21; pH 6.5, 0.10. The value of $\Delta\log\alpha^*$ for pH 2.6 (0.65, Table 4) therefore appears unrepresentative for unknown reasons.

Values of r^2 (Table 4) for S vs. Δt_R range from 0.01 to 0.38. These data suggest somewhat greater correlation between these two quantities for the anilines than for the benzoic acids, at intermediate

Table 4
Summary of gradient steepness-selectivity as a function of sample type and pH (ionizable samples)

Sample	T (°C)	Average $\Delta\log\alpha^*$ (b)			r^2
		Low T	High T	Average[d]	
Benzoic acids[e]	32.1, 50.9				
pH 2.6		0.20	0.21	0.21	0.01
pH 3.2		0.24	0.25	0.25	0.01
pH 3.7		0.22	0.16	0.19	0.13
pH 4.3		0.09	0.16	0.13	0.06
Anilinese	32.1, 50.9				
pH 2.6		0.61	0.69	(0.65)	0.08
pH 3.6		0.16	0.13	0.14	0.38
pH 4.6		0.17	0.23	0.20	0.24
pH 5.6		0.11	0.12	0.12	0.01
Basic drugs[g]	30, 48.5	0.37	0.35	0.36	0.18
Herbicide sample[h]	39.9, 48.4	0.20	0.22	0.21	0.46
Chlorophylls[i]	40, 60	0.27	0.07	0.17	0.08
rhGH peptides[j]	20, 60	0.33	0.30	0.31	0.04
rt-PA peptides[j]	40, 60	0.29	0.30	0.30	0.10
Cereal proteins[j]	50, 70	0.18	0.18	0.18	0.44
Average		0.25	0.24	0.24	
Average dev.[k]				0.03	

Same samples and conditions as Table 3. The correlation coefficient r^2 for the dependence of Δt_R on S is also given.
[a–k] See Table 3.

pH values ($3.6 \leq pH \leq 4.6$) where the sample is partially ionized. The greater correlation of aniline S and Δt_R values for intermediate pH-values is reminiscent of a pronounced correlation of %B- and pH-selectivities for these same compounds; see the discussion of Fig. 8 of [16] and note that %B-selectivity in isocratic separations is equivalent to b-selectivity in gradient separations. One possible explanation for this correlation of different selectivity effects is that changes in either %B (or b) or temperature cause a change in pK_a for the aniline solutes. In that case, both b-selectivity and T-selectivity would be correlated with pH, and the correlation of b- and T-selectivities would then be greatest when $pH \approx pK_a$.

3.2.3. Preferred mobile phase pH

The choice of a preferred pH for the optimal separation of acids and bases can now be considered. The conclusions reached for the benzoic acid and aniline samples should be applicable to other samples, if "low pH" results in the ionization of bases and the protonation of acids, and vice versa for "high pH" operation. It appears that temperature-selectivity will be greater for more ionized acidic samples, and greater for $pH \approx pK_a$ in the case of basic samples. From the standpoint of overall method development, however, $pH < 3$ is probably advantageous. Solvent-strength selectivity appears to be somewhat greater at low pH, and there is a greater correlation between b- and T-selectivity when $pH \approx pK_a$ (undesirable). A $pH < 3$ is also preferable for other reasons; silanol effects, which lead to band tailing and low plate numbers for basic samples, are minimized at low pH [17]. Likewise, a mobile phase $pH < 3$ minimizes day-to-day variations in the retention times of most acidic or basic solutes (whose pK_a values are usually >3), due to unavoidable small errors in mobile phase pH and the greater effect of these errors on retention when $pH \approx pK_a$. Finally, in Part I [11] it was found that predictions of retention as a function of temperature are less reliable for basic samples when $pH \approx pK_a$.

Some basic compounds used with certain columns

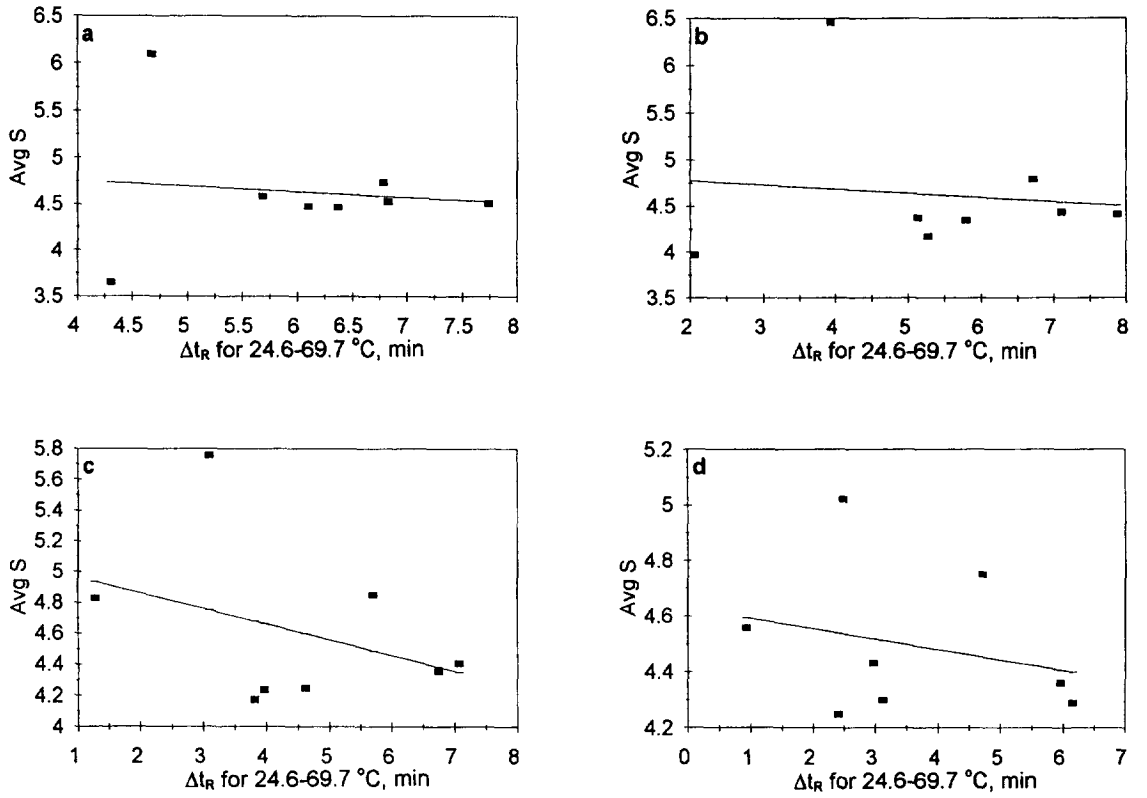


Fig. 3. Correlation of average values of S vs. values of Δt_R for benzoic acid sample (24.6–69.7°C). (a) pH 2.6; (b) pH 3.2; (c) pH 3.7; (d) pH 4.3. See Section 3.1.2 for details.

may give narrower bands and less tailing for pH>7 [18]. The data of Table 3 and Table 4 suggest that b- and T-selectivity effects will still be significant ($\Delta\log \alpha^* > 0.02$) for such samples at higher pH although this requires further verification.

Preferred, low pH conditions were used for the remaining four samples described below. These examples, along with the benzoic acids and anilines described above, allow a further test of the usefulness of temperature and gradient steepness as means for controlling band spacing at low pH.

3.3. Basic drug sample

The drug sample from laboratory B (Table 2 of Ref. [11]) includes 22 bases that are largely protonated at the pH of this study (pH≈2.4), as well as seven acids (protonated and neutral) and 18 neutral compounds. The 22 bases are emphasized here; the remaining 25 un-ionized acid and neutral compounds are discussed further in Part IV [12].

3.3.1. T- and b-selectivity

Fig. 4a plots values of Δt_R vs. t_R for this sample. The derived value of $\Delta\log \alpha^*(T)$ (Table 3) is 0.11, which can be compared with the value for the aniline sample at pH 2.6 ($\Delta\log \alpha^* = 0.07$). Fig. 4b plots S vs. t_R; a derived value of $\Delta\log \alpha^*(b) = 0.36$ was found (Table 4), vs. values for the aniline sample at pH 2.6 of 0.65 (ACN) and 0.14 (MeOH). The T- and b-selectivity effects are weakly correlated (Fig. 4c, $r^2 = 0.18$). The basic drugs sample thus confirms our findings for the benzoic acid and aniline samples: T- and b-selectivity are significant at low pH, with temperature being less important by a factor of about three.

Because of the large number of compounds in the total drug sample (47 vs. only 8–9 in the previous

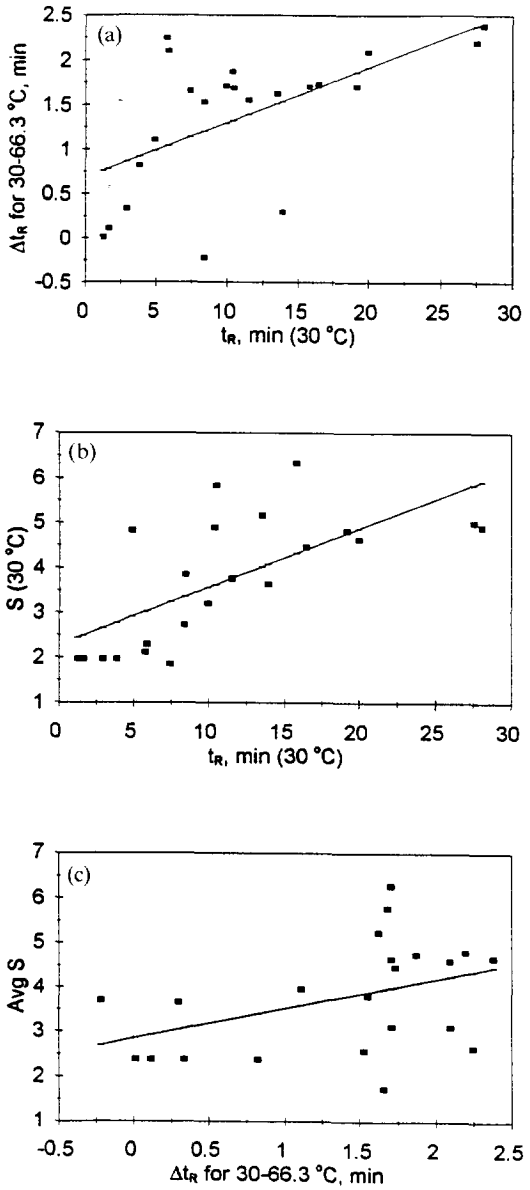


Fig. 4. Correlations for basic drugs sample (laboratory B). Data for 60-min gradient time; (a) correlation of Δt_R with t_R; (b) correlation of S with t_R; (c) correlation of average S with Δt_R (30–66.3°C). Unreliable S-values for early bands omitted from (b) and (c). See Section 3.3.1 for details.

two samples), T- and b-selectivity can be evaluated in an alternative, more illustrative fashion. Beginning with the 20-min gradient at 30°C, several incompletely resolved band-pairs ($R_s<0.9$) were identified, as summarized in Table 5. The effect of a change in temperature (from 30 to 66.3°C) or gradient steepness (from 20 to 60 min gradient time) on α and resolution for these band-pairs was examined. As seen in Table 5, an increase in temperature (66.3°C) resulted in much better separation of five of the eight band-pairs (1–3, 5, 7). Similarly, an increase in gradient time improved the separation of six band-pairs (2–6, 8). Using one variable or the other, it was possible to achieve baseline resolution ($R_s>1.5$) for all the band-pairs that were originally overlapped in the 30°C, 20-min run.

3.4. Herbicide sample

This sample consists of nine structurally-related impurities. It is therefore typical of many "real" samples; e.g. raw materials, degraded products, reaction mixtures, etc. Values of $\Delta\log\alpha^*$ are large for both gradient steepness (0.21, Table 4) and temperature (0.14, Table 3) selectivity effects. The apparent correlation of b- and T-selectivities is greater ($r^2=0.46$) than for the other samples, but this may be misleading. If the data point for compound 1 is deleted from the regression analysis, $r^2=0.03$.

Fig. 5 illustrates the effect of a 20°C change in temperature on the separation of the herbicide impurities. At first glance, temperature selectivity appears minor; i.e. there is no change in band elution order. However, this is a consequence mainly of the easy separation of this sample; the average resolution of the two runs is: $R_s=8.8$ (40°C) and 9.0 (60°C). More interesting is the average change in resolution ΔR_s for different band-pairs between the two runs: $\Delta R_s=1.9$ units, for only a 20°C change in temperature.

3.5. Chlorophyll sample

These seven plant pigments (Fig. 6) gave values of $\Delta\log\alpha^*$ that are similar to the average values of Table 3 and Table 4: $\Delta\log\alpha^*(T)=0.21$ and $\Delta\log\alpha^*(b)=0.17$. The two selectivities are only slightly correlated ($r^2=0.08$).

3.6. Peptide and protein samples

The separations of these three samples have been reported as a function of temperature and gradient

Table 5
Effect of a change in temperature or gradient steepness on resolution of drug sample (47 acids, bases and neutrals)

Band-pair[a]	R_s[b]		
	30°C, 20 min	66.3°C, 20 min	30°C, 60 min
1	0.4	**1.6**	0.8
2	0.2	**11.4**	**4.0**
3	0.7	**−1.8**	**−2.8**
4	0.8	−0.4	**−3.8**
5	0.7	**−5.4**	**3.7**
6	0.7	0.4	**6.6**
7	0.7	**2.9**	0.4
8	0.5	0.0	**1.4**
Average absolute change in R_s		3.0	2.9

Band-pairs that were incompletely resolved at 30°C with a 20-min gradient. Other conditions as in Ref. [11]. Improved separation (vs. 30°C, 20 min run) shown in bold.

[a] 1, morphine/5-hydroxyquinoline; 2, tranylcypromine/tripelennamine; 3, codeine/acetaminophen; 4, β-hydroxy theophylline/phentermine; 5, salicylic acid/butabarbital; 6, oxazepam/chlorpromazine; 7, flunitrazepam/lormetazepam; 8, phenylbutazone/mefenamic acid.

[b] Assumes average baseline bandwidth of 0.12 min for 20-min gradients, 0.25 min for 60-min gradients (experimental values at 30°C for C_3–C_{10} nitroalkanes); negative values of R_s indicate band reversal.

steepness [9,19]. In each case, gradients of 0–60% acetonitrile–water were used, with 0.1% of trifluoroacetic acid (TFA) added to each solvent. These peptides and proteins will have one or more ionized basic groups in each molecule under the conditions of separation (pH≈2). In addition, protonated carboxyl groups are also present in each molecule.

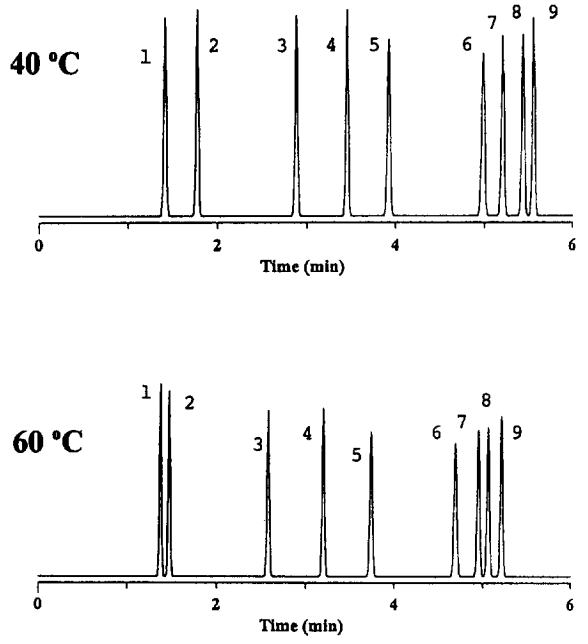


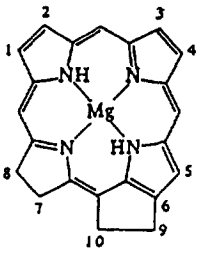
Fig. 5. Separation of herbicide sample at two different temperatures. Conditions: 7.5×0.46 cm, 3.5 μm Zorbax SB-C8 column; 5–95% B in 10 min; 2.0 ml/min; temperatures given in figure. Chromatograms are simulations based on experimental data of laboratory C.

3.6.1. rhGH tryptic digest

Values of $\Delta\log\alpha^*$ for this sample (for change in either T and b) are given in Table 3 and Table 4. Temperature and gradient steepness have a large and similar effect on $\Delta\log\alpha^*(b)=0.31$ and $\Delta\log\alpha^*(T)=0.35$, unlike the case of the previous samples where gradient-steepness effects were two to three times more important. Using temperature and gradient steepness optimization, it was possible to separate all 21 major peptides in the rhGH digest [19].

3.6.2. rt-PA tryptic digest

This sample contains 38 major peptide bands whose retention was studied. Values of $\Delta\log\alpha^*$ for a change in T or b are large and comparable to values for the rhGH digest (Table 3 and Table 4). The correlation of S and Δt_R values is weak ($r^2=0.10$), confirming the value of varying b and T together during method development. Because of the large number of compounds in the sample, it was not possible to separate every band in a single run. However, any individual peptide band could be



Compound	Substituents			
	-2	-3	-4	-7
Chlorophyllide a	$-CH=CH_2$	$-CH_3$	$-CH_2-CH_3$	$-CH_2-CH_2-COOH$
Chlorophyll C_3	$-CH=CH_2$	$-CO_2-CH_3$	$-CH=CH_2$	$-CH=CH-COOH$
Chlorophyll C_1	$-CH=CH_2$	$-CH_3$	$-CH_2-CH_3$	$-CH=CH-COOH$
Chlorophyll C_2	$-CH=CH_2$	$-CH_3$	$-CH=CH_2$	$-CH=CH-COOH$
Chlorophyll b	$-CH=CH_2$	$-CHO$	$-CH_2-CH_3$	$-CH_2-CH_2-COO-C_{20}H_{39}$
Monovinyl-chlorophyll a	$-CH=CH_2$	$-CH_3$	$-CH_2-CH_3$	$-CH_2-CH_2-COO-C_{20}H_{39}$
Divinyl-chlorophyll a	$-CH=CH_2$	$-CH_3$	$-CH=CH_2$	$-CH_2-CH_2-COO-C_{20}H_{39}$

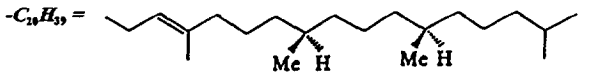


Fig. 6. Structures of the seven components of the chlorophyll sample.

resolved from all other bands in the sample [9] by optimizing temperature and gradient steepness.

3.6.3. Cereal protein sample

The separation of 15 major proteins in this sample was studied as a function of temperature and gradient steepness. Derived values of $\Delta\log\alpha^*$ in Table 3 and Table 4 indicate significant *b*- (0.18) and *T*- (0.38) selectivity, although the correlation of these two effects is greater ($r^2=0.44$) than observed for the other ionic samples of Table 4. All 15 proteins could be separated using an optimized temperature and gradient steepness [9].

The above peptide and protein samples exhibit larger values of $\Delta\log\alpha^*(T)$, compared to the smaller organic molecules in the benzoic acid, aniline and basic drug samples. This may be due to any of several factors: (a) the mobile phase for these separations contains trifluoroacetic acid, a weak ion-pair reagent (item 3 of Table 1), (b) peptides and proteins can adopt different conformations (secondary protein structure) under reversed-phase conditions (item 1 of Table 1) and (c) peptide and protein molecules usually have several ionic substituents, which may amplify selectivity effects due to the participation of each ionizable group.

3.7. Other observations from the data of Table 3 and Table 4

In Part II [1], we noted that the derivation of an equation for the measurement of $\Delta\log\alpha^*(T)$ values assumes that coefficient *B* does not vary systematically with %B. If *B* tends to increase for

smaller %B (as seems likely), larger values of $\Delta\log\alpha^*(T)$ should result for smaller %B and smaller values of b (larger values of t_G). This possibility is tested in Table 3 by comparing values of $\Delta\log\alpha^*(T)$ that are measured for different gradient times (low t_G vs. high t_G). The average value of $\Delta\log\alpha^*(T)$ for all samples is 0.20 for the low t_G runs and 0.17 for the high t_G runs. This is a small difference, probably within experimental error, but is in the opposite direction predicted by an increase in B for smaller %B.

The accuracy of values of $\Delta\log\alpha^*(T)$ as reported in Table 3 can be assessed by comparing values for the same sample measured with different values of t_G. The average deviation of such paired results for each compound from an average value of $\Delta\log\alpha^*(T)$ was 0.02 log units, which can be compared with an average value of $\Delta\log\alpha^*(T)$ for all samples of 0.18. We consider this agreement reasonable.

4. Conclusions

The present study of 119 ionizable compounds has shown that selectivity is strongly dependent on temperature (T) and gradient steepness (b). The two selectivities are correlated weakly, if at all, so that a simultaneous change of T and b appears generally well suited for the control of band spacing and resolution. T- and b-selectivities vary with sample ionization and, therefore, with mobile phase pH. T-selectivity is somewhat greater for partially-ionized basic samples (i.e. when mobile phase pH$\approx$pK_a for the sample), compared to samples that are unionized or completely ionized. However, b-selectivity appears to be somewhat greater at low pH. For this and other reasons, we recommend that initial separations be carried out at low pH, where most acidic and basic solutes will be protonated.

Previous studies (see Part II [1]) have established that b-selectivity is useful in controlling band spacing for a wide range of sample types; the present results indicate that T-selectivity should add significantly to overall selectivity for the case of any sample that contains ionizable compounds. Because of the somewhat greater importance of b-selectivity, it is advisable to begin method development by changing gradient steepness rather than temperature. If further changes in band spacing are required, changes in temperature can then be explored.

Temperature-related changes in band spacing are more pronounced for peptide and protein samples, compared to small ionizable compounds. Changes in gradient steepness and temperature are therefore strongly recommended for the control of band spacing for peptide and protein samples.

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