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Combined use of temperature and solvent strength in reversed-phase gradient elution
II. Comparing selectivity for different samples and systems

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Abstract

A change in temperature (T) or gradient steepness (b) can result in changes in reversed-phase selectivity (α). The magnitude of these changes in α will vary with other separation conditions (column, pH, etc.) and with sample type. In this paper, selectivity changes as a function of T and b are discussed and a simple treatment that allows changes in selectivity to be compared quantitatively for different samples and HPLC conditions is developed. Following papers in this series will apply this theory to arrive at conclusions concerning the use of temperature and gradient steepness in HPLC method development. The present treatment assumes that gradient-steepness selectivity (measured by the parameter S) does not change significantly with temperature. Data for a wide range of compound types and conditions are provided in support of this assumption.

Keywords: Selectivity; Column temperature; Gradient steepness; Gradient elution

1. Introduction

The preceding paper [1] has shown that computer simulation can be used to predict reversed-phase high-performance liquid chromatography (RP-LC) separation as a function of gradient conditions and temperature. If only gradient conditions are changed, selectivity (α) can vary significantly as a function of gradient steepness, b [2–8]. The potential for a change in temperature to affect selectivity in RP-LC is less clear. The conventional wisdom is that "...changes in sample resolution as a function of column temperature are fairly modest in most cases" [9]. However, there are numerous reports [10–30] that a change in temperature for either isocratic or gradient HPLC separation can lead to useful changes in α. A few studies describe the use of temperature and solvent strength (gradient steepness or isocratic %B) in combination to control band spacing and optimize resolution [8,16,21,25].

It is difficult on the basis of prior literature to determine the general usefulness of temperature optimization for HPLC method development. It is not known how temperature selectivity varies with sample type and it is also unclear how other separation conditions (pH, column type, etc.) affect temperature selectivity. Table I of Part III [31] summarizes some phenomena that can, in principle,

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lead to temperature-induced changes in selectivity. Perhaps temperature optimization would be more effective if (a) applied only to certain samples and (b) other separation conditions were chosen to maximize changes in α with temperature. This paper describes a quantitative basis for assessing the relative importance of both gradient-steepness and temperature selectivity for different samples in different gradient separations. This procedure is applied in Parts III and IV of this series [31,32] to evaluate temperature selectivity as a function of sample type and other separation conditions.

2. Theory

For terms defined here and in Part I [1], see the Glossary of Terms in Ref. [1].

2.1. Solvent strength selectivity

A theory of solvent strength selectivity (change in α with either b or %B) has been described for both isocratic [33] and gradient [2,3] elution. This theory will be reviewed and extended here as a basis for, and comparison with, the following discussion of temperature selectivity.

2.2. Isocratic elution

When solvent strength (%B) is varied in isocratic elution (other conditions remaining constant), solute retention is related to the volume fraction of the B-solvent (ϕ=0.01%B) as [34]

$$\log k = \log k_w - S\phi \quad (1)$$

(same as Eq. 1 of Part I [1]). The linear relationship of log k vs. ϕ predicted by Eq. (1) is illustrated in the hypothetical examples of Fig. 1. Fig. 1a shows plots for the various components of a "regular" sample, where individual curves do not intersect each other. Mixtures of homologues [36,37], benzologues [13] or oligomers composed of molecules with repeating, identical units [38] usually behave as "regular" samples. Because the plots of Fig. 1a diverge as ϕ decreases, α increases for smaller ϕ in these examples. However, if two sample bands in a "regular" sample co-elute for some value of ϕ, they will co-elute for all values of ϕ. By our (arbitrary) definition, solvent strength selectivity does not exist for "regular" samples.

Fig. 1b shows similar plots for an "irregular" sample, where (if ϕ is changed sufficiently) individual curves may intersect, leading to retention

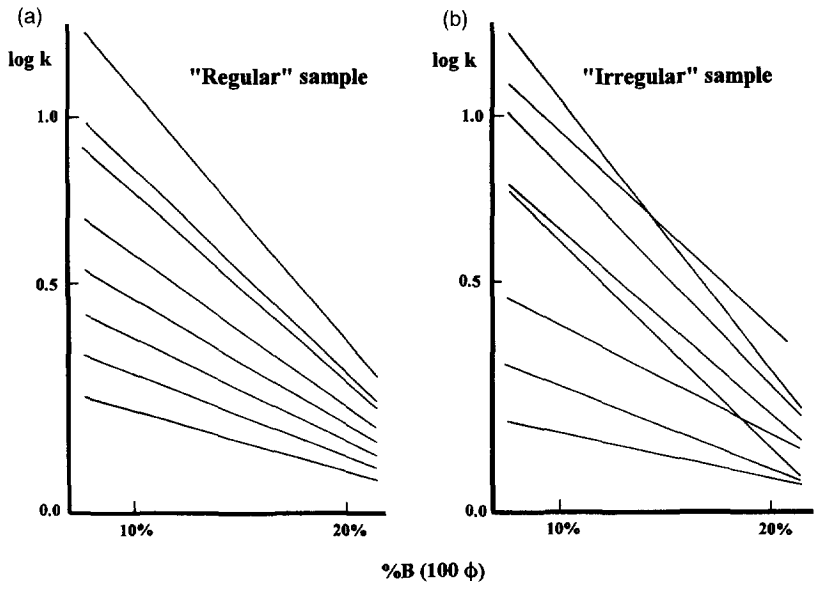

Fig. 1. Illustration of (a) "regular" vs. (b) "irregular" sample behavior for solvent-strength selectivity. See text for details.

reversals. An "irregular" sample may not appear to exhibit intersections, as in Fig. 1b, over some practical range in ϕ, but a change in ϕ may still result in significant changes in band spacing and resolution. A quantitative test of the significance of solvent-strength selectivity for a given sample (only ϕ varying) can be obtained as follows. The separation factor α for two solutes, 1 and 2, as a function of ϕ can be derived from Eq. (1):

$$\log \alpha = \log(k_2/k_1) = \log(k_{w_2}/k_{w_1}) - (S_2 - S_1)\phi$$
$$= \text{constant} - \Delta S\ \phi \qquad (2)$$

The solvent-strength selectivity or change in α as a result of a change in ϕ from mobile phase a′ to mobile phase b′ is then

$$\log \alpha_b - \log \alpha_a = \Delta \log \alpha = (S_2 - S_1)(\phi_b - \phi_a)$$

or

$$\Delta\log \alpha = \Delta S\ \Delta\phi \qquad (3)$$

The quantity $\Delta\log\ \alpha$ is a quantitative measure of solvent strength selectivity for isocratic elution. Values of $\Delta\log\ \alpha > 0.02$ (5% change in α) will prove useful for changing band spacing and improving resolution during method development.

2.3. Gradient elution

In gradient elution, mobile phase composition (%B) changes during the separation and k for each solute band also varies with time. It can be shown, however, that there is an average or effective value of k (k^*) for each band in a gradient chromatogram; k^* is the value of k for a band when it has migrated halfway through the column. This average retention, k^*, can be related to the conditions of separation [35]:

$$k^* = 0.87\ t_G\ F/(V_m\ \Delta\phi\ S) \qquad (4)$$

where t_G refers to gradient time, F is the flow-rate, V_m is the column dead-volume and $\Delta\phi$ is the change in ϕ during the gradient. The quantity k^* is also given by

$$\log k^* = \log k_W - S\phi^* \qquad (5)$$

where ϕ^* is the value of ϕ at the column midpoint at the time the band has reached the column midpoint (and $k = k^*$). The values of the parameters k_W and S in Eq. (5) for gradient elution are the same as for isocratic elution (Eq. (1); [8, 17, 18]). Therefore, k in Fig. 1 can be replaced by k^*, and ϕ by ϕ^*; i.e., the same plots apply for both isocratic and gradient elution.

Similarly, we can write (cf. Eq. (3))

$$\Delta\log \alpha^*(b) = \Delta S\ (\phi_a - \phi_b) = \Delta S\ \Delta\phi^* \qquad (6)$$

The quantity $\Delta\log\ \alpha^*(b)$ refers to a change in $\log\ \alpha^*$ as a result of a change in gradient steepness b. In isocratic elution, it is desirable that $1 < k < 10$ for all bands in a chromatogram. Similarly, in gradient elution, conditions should be selected for $1 < k^* < 10$. From Eq. (5), the latter condition corresponds to $\Delta\phi^* < 1/S$, which, with Eq. (6), yields

(gradient elution, $1 < k^* < 10$)

$$\Delta\log \alpha^*(b) = 1.0\ \Delta S/S. \qquad (7)$$

A change in k^* by $10/1 =$ ten-fold can be achieved by a change in gradient time, t_G, by a factor of ten (Eq. (4)). When there are more than two sample components, it is unlikely that k in isocratic elution can be changed ten-fold while maintaining $1 < k < 10$ for all bands. This means that solvent-strength selectivity will be more effective in gradient elution, compared to isocratic separation.

2.4. Quantitative evaluation of solvent-strength selectivity

How large a change in α can be expected when ϕ is changed for a typical example? The isocratic example of Fig. 1 will be used to answer this question. In Fig. 2, values of S are plotted vs. t_R for each sample (data of Fig. 1). The regular sample (Fig. 2a) has S values that correlate closely with retention time; the deviation of points from the best curve through these data is minimal. Therefore, two compounds that overlap completely will have the same retention time and the same value of S. Two such compounds cannot be separated by changing %B.

The irregular sample (Fig. 2b) shows a different pattern; values of S deviate significantly from the best-fit curve (not necessarily a straight line) through these data. This deviation of S (1 S.D.) for an

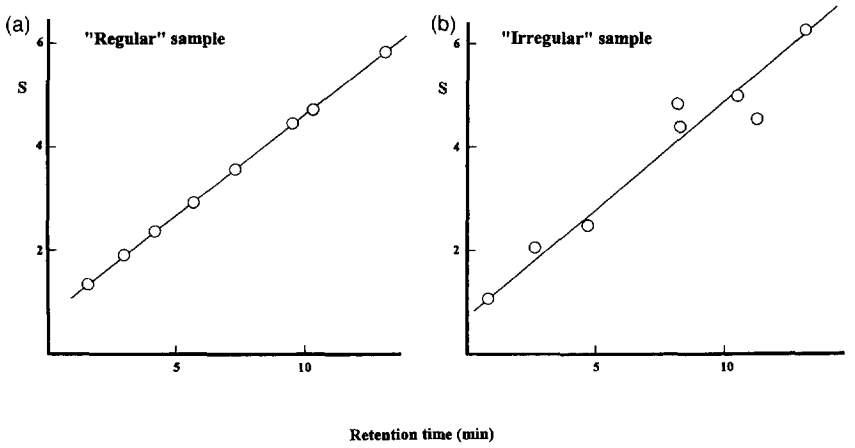

Fig. 2. Quantitative determination of the extent of solvent-strength selectivity. (a) "Regular" sample; (b) "irregular" sample. See text for details.

average solute will be defined as $\delta S = [\Sigma x^2/(n-1)]^{1/2}$, where values of x are the differences between actual and best-fit values of S, and n is the number of data points (values of S). The effective value of ΔS (1 S.D.) in Eq. (7) for two adjacent bands is then $2^{1/2}\ \delta S$, or (Eq. 1)

$$\Delta \log \alpha^*(b) = 1.4\ \delta S/S. \tag{7a}$$

In the hypothetical example of Fig. 2b, δS is 0.5 units and the average value of S is 4.5, so $\delta S/S = 0.5/4.5 = 0.11$. The average change in log $\alpha^*(b)$ for a change in t_G by ten-fold is therefore 0.15 (Eqs. (7a)), corresponding to a change in α of 40%. This is a greater change in α than will normally be required in method development (the "irregular" sample used as an illustration in Fig. 1a and Fig. 2a is deliberately extreme). Note also that greater solvent-strength selectivity, which is our goal in method development, corresponds to increased scatter of plots, as in Fig. 2b.

2.5. Temperature selectivity

Temperature selectivity can be discussed in a similar fashion as for solvent-strength selectivity. With few exceptions [39–42], isocratic retention as a function of temperature is accurately described by the van't Hoff relationship (see Part I and Ref. [17])

$$\log k = \mathrm{A} + \mathrm{B}/T \tag{8}$$

where A and B are constants for a given compound and set of experimental conditions and T is the absolute temperature. Replacing the x-axis variable in Fig. 1 by $1/T$ would result in similar (linear) plots for retention as a function of temperature, and "regular" and "irregular" samples can be defined for temperature selectivity in the same way as solvent-strength selectivity in the preceding discussion (see Fig. 1 of Ref. [6]).

A quantitative test for the significance of sample "irregularity" or temperature selectivity can be developed paralleling that for solvent-strength selectivity. The separation factor for two adjacent bands can be derived from Eq. (8) (cf Eq. (2)):

$$\log \alpha = \log(k_2/k_1) = (\mathrm{A}_2 - \mathrm{A}_1) - (\mathrm{B}_2 - \mathrm{B}_1)(1/T)$$
$$= \text{constant} + \Delta\mathrm{B}(1/T) \tag{9}$$

The change in α as a result of a change in temperature from T_a to T_b is then (cf. Eq. (3))

$$\Delta \log \alpha(T) = \Delta\mathrm{B}\ \Delta(1/T) \tag{10}$$

The quantity $\Delta \log \alpha(T)$ is a quantitative measure of temperature selectivity for isocratic elution.

2.6. Gradient elution

A quantitative measure of temperature selectivity can be derived for gradient elution, parallel to the above case for isocratic separation and to the prior derivation of solvent strength selectivity in gradient elution. From the Appendix A, we have (cf. Eq. (10) for isocratic elution)

$$\Delta\log \alpha^* = \Delta B\, \Delta(1/T) \quad (11)$$

which can be compared to Eqs. (6,10). Also (Appendix A),

$$B = (b/t_0)\, \Delta t_R/\Delta(1/T) \quad (12)$$

Values of B or Δt_R can be plotted vs. t_R (T constant) for different solutes as a test of sample "regularity" when the temperature is changed. This is similar to the procedure of Fig. 2, which plots values of S vs. t_R as a test of "sample regularity" when %B is changed. As in the case of Fig. 2b, increased scatter of plots of Δt_R vs. t_R favors HPLC method development based on changes in T.

There is no inherent limit on how much temperature can be changed in RP-LC; temperatures <0 and >100°C have been used. For "every day" application, however, it is reasonable to restrict temperature within 30 and 90°C. This results in a maximum value of $\Delta(1/T)\approx 0.0005$. Eq. (10) for isocratic elution then becomes

$$\Delta\log \alpha^*(T) \approx 0.0005\, \Delta B \quad (13)$$

The quantity $\Delta\log \alpha^*(T)$ is the possible change in $\log \alpha^*$ as a result of a change in temperature. In following papers [31,32], we have evaluated temperature selectivity by plotting values of Δt_R vs. t_R, and measuring the deviation $[\delta(\Delta t_R)]$ of individual values of Δt_R (1 S.D.) from a best-fit curve through these data (similar to the determination of values of δS described above). A value of δB can be calculated (Eq. (12)) from $\delta(t_R)$ as

$$\delta B = (b/t_0)\, [\delta(\Delta t_R)]/\Delta(1/T) \quad (14)$$

The average value of ΔB in Eq. (13) will then be $2^{1/2}\ \delta B$. Eq. (13) thus allows a quantitative comparison of temperature selectivity for different samples and different conditions in terms of values of $\Delta\log \alpha^*(T)$.

3. Experimental

See Part I [1] for data from laboratories A–E.

4. Results and discussion

4.1. Values of S as a function of temperature

Eq. (12) (as well as the derivation of Eq. 11 of Part I) assumes that S does not change when T is varied. This has been further confirmed in the present study (data of [1,31,32]) as summarized in Table 1. Fig. 3 illustrates the constancy of S as T is varied for eight homologous nitroalkanes (a) and 40 miscellaneous drugs (b). Values of S measured at 66.3°C are plotted vs. values measured at 30°C, and the line $y=x$ through these data provides a close fit. In other cases, small decreases in S at higher temperatures have been reported [34,43]. Table 1 lists the ratio (S_a/S_b) of average values of S at two temperatures T_a and T_b for different samples and under HPLC conditions studied by us or reported previously. Values of $(S_a/S_b)\approx 1.0$ for all cases, except for the aniline sample at pH 3.6 (where $\mathrm{pH}\approx \mathrm{p}K_a$). For this one case, involving partially ionized bases, S decreased significantly at higher temperatures. There is also considerable scatter in the plot of S (69.7°C) vs. S (25.5°C) for the anilines at pH 3.6. All the examples but one of Table 1 involve acetonitrile as solvent B.

Eqs. (11,12) also assume that the value of the coefficient B does not change as ϕ is varied, which should be the case if S is not a function of T. In Parts III and IV [31,32], this question is dealt with further: Values of $\Delta\log \alpha^*(T)$ are measured for two different gradient times t_{Ga} and t_{Gb} and compared. A change in t_G causes a solute band to elute at a different value of ϕ, so a comparison of values of $\Delta\log \alpha^*(T)$ vs. t_G provides some measure of the uncertainty of these experimental values of $\Delta\log \alpha^*$(T) as a result of the variation of B (or S) with T.

Table 1
Variation of S with temperature

Laboratory	Sample	Conditions	T_a, T_b (°C)	S_b/S_a
A	Benzoic acids	pH 2.6	24.6, 69.7	0.95
		pH 3.2		0.98
		pH 3.7		1.00
		pH 4.3		0.92
A	Anilines	pH 2.6	25.5, 69.7	0.92
		pH 3.6		0.83[a]
		pH 4.6		1.00
		pH 5.6		0.94
B	Nitroalkanes		30, 66.3	1.03
B	Drugs		30, 66.3	0.99
C	Herbicides		40, 60	1.03
D	Pharmaceuticals		35, 75	0.97
E	PAHs		25, 45	1.03[b]
F	Fatty acid methyl esters		30, 70	1.07[c]
	rhGH Peptides [39]		20, 60	1.04
	rt-PA Peptides [8]		40, 60	0.95
	Cereal proteins [14]		50, 70	1.14[a]
		Average		0.98±0.06[d]

Data are taken from Parts I, III and IV [1,31,32]. T_a and T_b refer to the lower (a) and higher (b) temperatures. The ratio S_b/S_a is the averge ratio of corresponding S values.
[a] Values of S_b/S_a vary considerably for different solutes (these samples were therefore not included in the overall average value for $S_b/S_a = 0.98$).
[b] Average value for two different columns.
[c] Average value for methanol and acetonitrile solvents.
[d] Excluding value for pH 3.6 anilines (see note [a]).

4.2. Quantitative evaluation of temperature and gradient-steepness selectivity

Data from a previous study [44] will be used to illustrate our approach to evaluating temperature and gradient-steepness selectivity effects. The sample of [44] is a tryptic digest of recombinant human growth hormone (rhGH). Retention times were determined for gradient times of 30, 60 and 120 min, and for temperatures of 20, 40 and 60°C. Using these data, values of S were measured for each temperature and averaged for each peptide (Table 2 of Ref. [44]). Similarly, values of Δt_R were calculated for a change in temperature from 60 to 20°C for each gradient time t_G, adjusted for differences in t_G (Eq. (12)) and averaged for each peptide (Table 4 of Ref. [44]).

Fig. 4a is a plot of average values of S for each peptide vs. retention time (20°C with $t_G = 120$ min). If solvent-strength selectivity was negligible for this sample, a smooth curve would connect all the data points. The scatter actually observed for this data set indicates significant solvent-strength selectivity, which will benefit method development. Values of S tend to be less reliable for solutes that elute early (small k_0), so S-values for the first two peaks are excluded from the correlation of S vs. t_R in Fig. 4a; see the further discussion of Ref. [44]. The average deviation of values of S from the solid line of Fig. 4a is $\delta S = \pm 4.6$ (1 S.D.), the average value of S is 21.4 and $\delta S/S = 4.6/21.4 = 0.21$. The average value of $\Delta\log\ \alpha^*(b)$ that can be achieved by a ten-fold change in gradient steepness is then $2^{1/2}(\delta S/S) = 0.30$ (Eq. (7)). It was established in [44] that band spacing for this sample is highly dependent on gradient steepness, in agreement with the latter large value of $\Delta\log\ \alpha^*(b)$.

Fig. 4b plots average values (adjusted for $t_G = 120$ min) of Δt_R vs. t_R. The deviation of values of Δt_R from the best-fit solid curve is ±1.9 min (1 S.D.). Based on gradient conditions and an average value of $S = 22$, the average value of $(b/t_0) = 0.110$. The average value of $\Delta\log\ \alpha^*(T)$ that is possible for a 60°C change in temperature (based on a 40°C change in Fig. 4b) can be calculated from Eqs. (12,13):

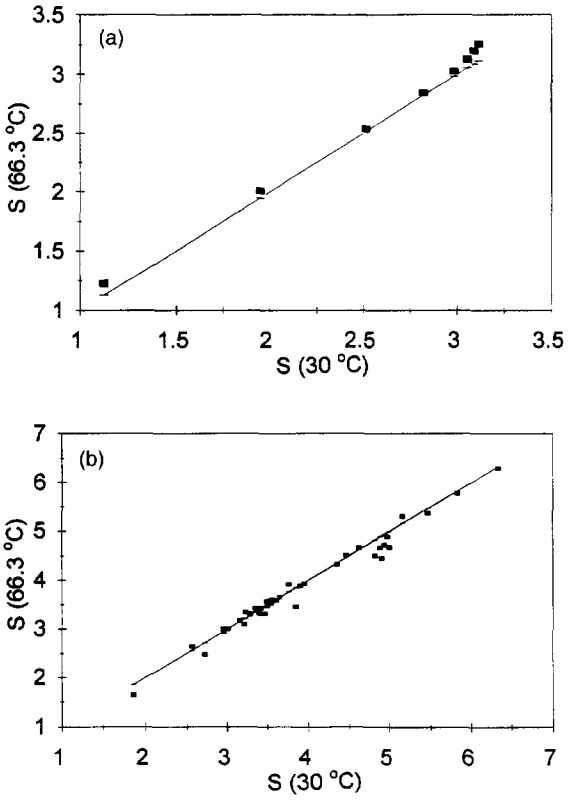

Fig. 3. Constancy of S as temperature is varied [values of S for 66.3°C (y) and 30°C (x)]. (a) Nitroalkane sample from Laboratory B; (b) drug sample from Laboratory B. Solid curves are $y=x$.

$\delta B=509$ and $\Delta\log\ \alpha^*=0.36$. Note in this case that temperature selectivity [$\Delta\log\ \alpha^*(T)=0.36$] is comparable to gradient-steepness selectivity [$\Delta\log\ \alpha^*(b)=0.30$]. A change in either variable could be expected to cause large changes in band spacing.

In Fig. 4c, average values of S (data for 20, 40 and 60°C), which measure b-selectivity, are plotted vs. average values of Δt_R for a change in temperature from 60 to 20°C (which measures temperature selectivity). If similar changes in selectivity resulted from a change in either gradient steepness or temperature, these data should fall close to a smooth curve. Fig. 4c shows that this is not the case; rather, changes in α due to b or T (for this sample) are uncorrelated ($r^2=0.04$), meaning that these variables affect selectivity differently and will be complementary during method development. This has been confirmed previously [44], where it was found that separation of all

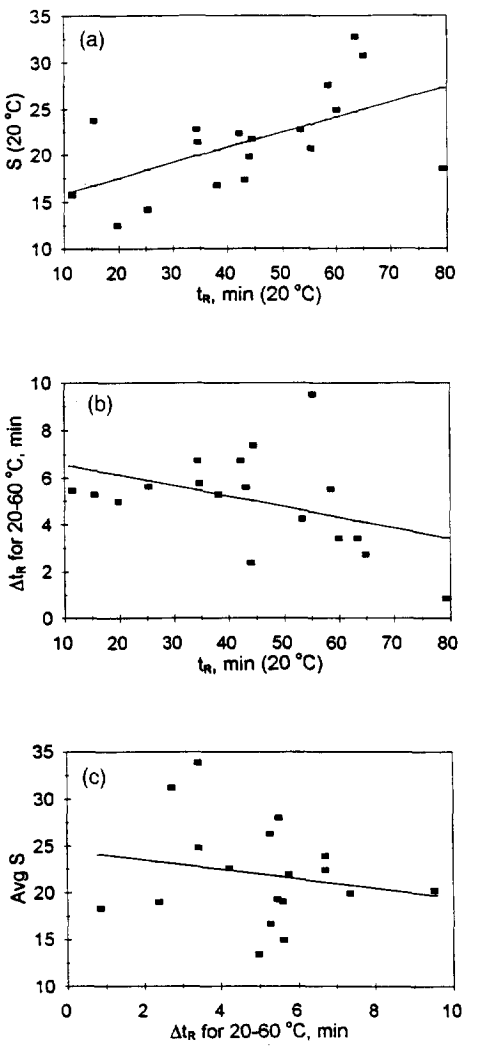

Fig. 4. Selectivity effects for the peptides in an rhGH protein digest. (a) Values of S (20°C) plotted against retention time ($T=20$°C, $t_G=120$ min); (b) average (adjusted) values of Δt_R plotted against retention time ($T=20$°C, $t_G=120$ min); (c) average values of S plotted against average values of Δt_R. Data from Ref. [44].

21 peptides of the rhGH digest was possible in a single separation that optimized b and T together. An absence of correlation, as in Fig. 4c, will always confirm an independence of gradient-steepness and temperature selectivity effects. For some samples, however, both S and Δt_R each tend to correlate with retention time (as in Fig. 2a for S), so that a correlation of S and Δt_R might in some cases mask

the independence (non-correlation) of b- and T-selectivities. For this reason, a better test of correlation of b- vs. T-selectivities might be to plot values of δS vs. $\delta(\Delta t_R)$, instead of S vs. Δt_R. This was investigated for the studies of Parts III and IV [31,32], but in every case, there was little difference between values of r^2 for either correlation. Values of r^2 reported in Parts III and IV are based on correlations of S vs. Δt_R.

4.2.1. Possible problems of interpretation

The data of Fig. 4a,b are each fit by a linear curve in order to extract values of δS and $\delta(t_R)$ for the calculation of values of $\Delta\log \alpha^*$. In some cases, it is apparent that the best fit of values of S or Δt_R vs. t_R is given by a curvilinear relationship. This is illustrated in Fig. 5 (similar plots as in Fig. 4) for the nitroalkane sample of Laboratory B (defined in Part I [1]). The nitroalkane sample is a mixture of homologues and should therefore be "regular", i.e., no selectivity is expected due to temperature or gradient steepness. However, the best straight-line fit (dashed curve) in each case results in significant values of δS and δt_R (deviations of data points from the dashed line), suggesting significant b- and T-selectivities for these "regular" homologues. The reason for the latter (incorrect) conclusion is that a smooth curve can (and should) be used to connect all the data points in each plot, as shown by the solid curves through each data set. In other cases examined by us (Parts III and IV [31,32]), involving "irregular" samples, it was possible to use a two-segment linear curve as a reasonable fitting curve when a linear fit appeared inappropriate. Due to the scatter of the data of Fig. 4a,b from any best-fit curve, resulting values of δS or δB are not much different for curved vs. straight-line plots. A straight-line plot will, in most cases, adequately correct resulting values of δS or δB for "regular-sample selectivity".

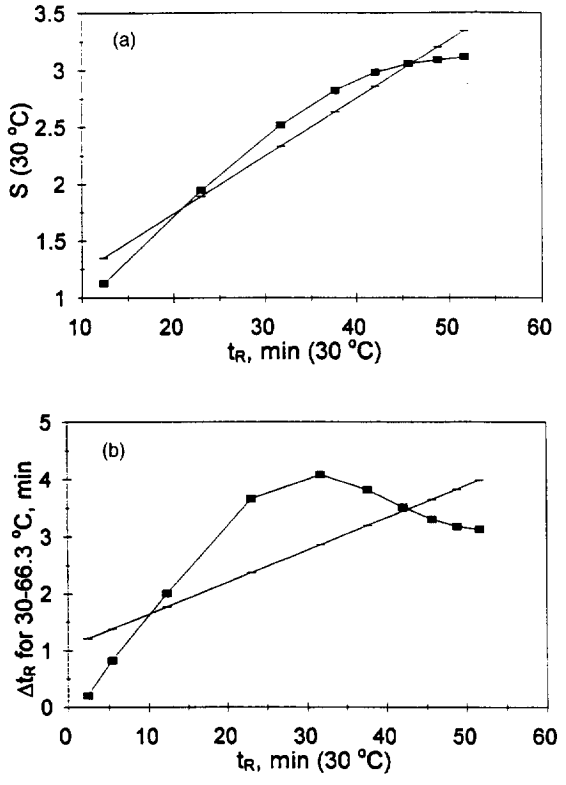

Fig. 5. Non-linear dependence of S and Δt_R vs. t_R for an homologous sample (nitroalkanes from Laboratory B). (a) Values of S at 30°C vs. t_R at 30°C (60 min gradient); (b) values of Δt_R (30°C values minus 66.3°C values) vs. t_R at 30°C. Curved line is best fit to data; straight line is linear best-fit.

5. Conclusions

Changes in α as a result of a change in temperature T or gradient steepness b (equivalent to isocratic %B) arise for both "regular" samples, such as homologues, and "irregular" samples, where changes in relative band position occur as T or b is varied. Such changes in selectivity for "regular" samples are of little value for the separation of overlapping bands, which represents the primary example where a change in α is needed. In the present paper, a treatment is presented which allows the derivation of average changes in selectivity ($\Delta\log \alpha$) for allowable changes in either solvent strength (b or %B) or temperature. These values of $\Delta\log \alpha$ are corrected for changes in selectivity of the "regular sample" type and are therefore more useful as measures of the ability of a change in solvent strength or temperature to provide useful changes in selectivity (i.e., for overlapping bands at one value of b or T). Following papers (Parts III and IV) use this approach to determine values of $\Delta\log \alpha$ (variation of either b or T) for a number of widely different samples. In this way, the utility of a change in b or T

for the purpose of changing α can be evaluated as a function of sample type.

Appendix A

Derivation of temperature selectivity in gradient elution.

In gradient elution [35], retention time t_R for well retained solutes is given as

$$t_R = (t_o/b) \log(2.3\, k_o b) + t_o + t_D \quad \text{(I–1)}$$

where k_o is the value of k at the start of the gradient and

$$b = V_m\, \Delta\phi\, S/(t_G F) \quad \text{(I–2)}$$

V_m is the column dead-volume (ml), $\Delta\phi$ is the change in ϕ from the start to the end of the gradient, t_G is gradient time (min) and F is flow-rate (ml/min). The quantities t_o and t_D are the column dead-time and gradient dwell time, respectively.

The retention times of a compound at temperatures T_a and T_b are (Eqs. (I–1))

$$t_{Ra} = (t_o/b_a) \log(2.3\, k_{oa} b_a) + t_o + t_D \quad \text{(I–3)}$$

and

$$t_{Rb} = (t_o/b_b) \log(2.3\, k_{ob} b_b) + t_o + t_D \quad \text{(I–3a)}$$

Previous studies [34] with work summarized in the present paper suggest that in most cases S varies only slightly with temperature. If it is assumed that S is constant for a compound as only temperature is varied, then b is also independent of temperature (Eqs. (I–2)). The change in retention time Δt_R for a change in temperature is then (Eqs. (I–3), I–3a)

$$\Delta t_R = (t_o/b) \log(k_{ob}/k_{oa}) \quad \text{(I–4)}$$

Combining Eq. (8) and Eqs. (I–4) then yields

$$\Delta t_R = (t_o/b)\, B\, [(1/T_a) - (1/T_b)]$$

or

$$B = \frac{(b/t_o)\, \Delta t_R}{[(1/T_a) - (1/T_b)]} = (b/t_o)\, \Delta t_R/\Delta(1/T) \quad (12')$$

As in our discussion of solvent-strength selectivity in gradient elution, a change in α as a result of a change in temperature can be derived (cf. Eqs. (6,10))

$$\Delta \log \alpha^*(T) = \Delta B\, \Delta(1/T) \quad (11')$$

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