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Combined use of temperature and solvent strength in reversed-phase gradient elution
IV. Selectivity for neutral (non-ionized) samples as a function of sample type and other separation conditions

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Abstract

The separation of nine un-ionized samples was studied as a function of temperature (T) and gradient steepness (b). Selectivity values $\Delta\log\alpha^*$ were obtained for 160 compounds, ranging from nonpolar hydrocarbons to very polar drugs. Selectivity varied markedly with sample type: nonpolar compounds such as aromatic hydrocarbons and fatty acid methyl esters generally showed only modest changes in band spacing as temperature or gradient steepness was varied. More polar samples exhibited larger changes in α ($\Delta\log\alpha^*$) when temperature and/or gradient steepness were changed, but the largest values of $\Delta\log\alpha^*$ for these non-ionized samples are less than the average value of $\Delta\log\alpha^*$ for the ionized samples of Part III [1]. Poly-functional silane ("polymeric") columns exhibit slightly increased b- and/or T-selectivity for some samples.

Keywords: Column temperature; Selectivity; Gradient elution; Gradient steepness; Polynuclear aromatic hydrocarbon; Nitroaromatics; Fatty acid methyl ester; Carotenoids; Pharmaceuticals; Non-basic drugs

1. Introduction

In Part III [1], temperature (T) and gradient–steepness (b) selectivity was investigated for acidic and basic samples under conditions where the solutes were ionized. In the present paper, a similar study is described for neutral and un-ionized compounds and compared with data for some protonated acids and unprotonated bases from Part III [1]. Studies of temperature selectivity as a function of solute molecular structure and other conditions have been reported for several different neutral compound types:

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polycyclic aromatic hydrocarbons (PAHs) [2–6], nitroaromatics [7,8], carotenoids [9,10], and fatty acid methyl esters [11–13]. Some of these reports have included information on the simultaneous effect of solvent strength (%B or b) and temperature on selectivity: PAHs [6], nitroaromatics [7,8] and fatty acid methyl esters [13].

Temperature selectivity has been examined in the most detail for the PAHs. It has been proposed [6] that T-selectivity for these compounds is determined mainly by their molecular shape and their relative ability to fit into narrow cavities or "slots" in the reversed-phase bonded phase. It has been shown that polymeric alkyl-silane bonded phases are more selective for molecules of different shape, and a change in temperature can to some extent duplicate a change in column type. That is, selectivities due to column type and temperature are strongly correlated. This suggests that the type of column used can affect how well temperature optimization will work for the PAH samples and perhaps other neutral samples whose components differ in shape.

The goals of this investigation were similar to those described in Part III [1] for ionizable samples: to determine solvent strength (b) and temperature (T) selectivities for a wide range of un-ionized samples as a function of other separation conditions (in the present case, column type).

2. Experimental

Equipment and procedures for laboratories A–D are described in Part I [14].

2.1. Laboratory E (PAH sample, Sander)

The equipment and procedures used are described in Ref. [4] and Part I [14]. A 16-component PAH sample (designated as priority pollutants by the Environmental Protection Agency) was used as sample by laboratory E (Table 1). Two different 25×0.46 cm columns were used: (a) Hypersil Green PAH (Shandon), referred to here as "polymeric column-1" and (b) Zorbax-ODS (Rockland Technologies, Newport, DE), referred to here as "monomeric column". All separations were carried out with a 40–100% acetonitrile (ACN)–water gradient at 1.0 ml/min with gradient times of 30 and 90 min, and temperatures of 25 and 45°C. Retention data for solutes eluting after the end of the gradient were omitted from further consideration.

Table 1
Components of the PAH sample (laboratory E and [17])

1	Naphthalene
2	Acenaphthylene
3	Acenaphthene
4	Fluorene
5	Phenanthrene
6	Anthracene
7	Fluoranthene
8	Pyrene
9	Benz[a]anthracene
10	Chrysene
11	Benzo[*b*]fluoranthene
12	Benzo[*k*]fluoranthene
13	Benzo[*a*]pyrene
14	Dibenz[*a,h*]anthracene
15	Benzo[*ghi*]perylene
16	Indeno[1,2,3-*cd*]pyrene

Some of these compounds did not elute during the gradients carried out at laboratory E and were therefore not used in calculations of $\Delta\log\alpha^*$ (11–16 for the monomeric column and 9–16 for the polymeric column). Separations were also carried out at 0°C, but these data were not used in this study because of late-elution of a majority of the compounds.

2.2. Laboratory F (fatty acid sample, Lin)

Equipment and procedures are described in Ref. [15]; a column heater was used to control temperature (set points only, no measurements), and the system dwell volume was 4.1 ml. The components of this sample are listed in Table 2. Two different 25×0.46 cm columns were used: (a) a monomeric column, Zorbax SB-C18 (Rockland Technologies) and (b) a polymeric column, Vydac 201TP (Separations Group). Separations were carried out with both acetonitrile–water and methanol–water gradients at 1.0 ml/min. Gradient times were 30 and 90 min, at temperatures of 30 and 70°C for column (a) and 40 and 70°C for column (b).

Data for the isocratic separation of a similar fatty acid sample as a function of %ACN–water and temperature have been reported [11], using a Polymicro capillary column. The sample is described in Table 3. It was also possible to derive b- and T-selectivity values for this column [12].

Table 2
Components of the Lin fatty acid methyl ester sample (laboratory F)

Fatty acid	Comment[a]
1. Ricinoleic	9-ene, 12-OH C_{18}
2. Vernolic	9-ene,12,13-epoxy C_{18}
3. Lesquorolic	9-ene, 12-OH C_{20}
4. Linolenic	9,12,15-tri-ene C_{18}
5. Palmitoleic	9-ene C_{16}
6. Stearolic	9-yne C_{18}
7. Linoleic	9,12-di-ene C_{18}
8. Malvalic	8-ene C_{19}[b]
9. Palmitic	C_{16}
10. Oleic	9-ene C_{18}
11. cis-7-Octadecenoic	7-ene C_{18}
12. Petroselenic	6-ene C_{18}
13. Elaidic	9-ene(*t*) C_{18}
14. Stearic	C_{18}

[a] "ene" refers to a *cis*-double bond at the indicated position (unless indicated as *trans* by *t*).
[b] Malvalic acid is a C_{18} acid with the eight and nine carbons connected into a cyclopropenyl ring.

2.3. Laboratory G (carotenoids sample, Van Heukelem)

The equipment (HP 1090 system with mobile phase preheating) and procedures used have been described [9]; the system dwell volume was 1.0 ml. The 15×0.46 cm column was packed with a custom C_{30} phase developed for the improved separation of very long molecules such as the carotenoids (courtesy of L. Sander). Two solvent-systems were employed. Mobile phase 1 used a gradient of 0–28% B in times of 25 and 75 min, with a flow-rate of 0.8 ml/min. The A-solvent was 90% methanol (MeOH)–0.5 *M* aqueous ammonium acetate (AmAc); the B-solvent was methyl-*tert.*-butylether (MTBE).

Separations were carried out at 40 and 60°C. Mobile phase 2 used a gradient of 0–100% B in times of 25 and 75 min, with a flow-rate of 0.9 ml/min. The A-solvent was 67:25:8 (v/v) MeOH–AmAc–MTBE; the B-solvent was 70:29:1 (v/v) MeOH–MTBE–AmAc. Data were collected at 45 and 55°C.

The sample structures are shown in Table 4.

Table 3
Components of the McGuffin fatty acid methyl ester sample [11]

Fatty acid	Comment[a]
1. Lauric	12:0; C_{12}
2. Myristoleic	14:1; 9-ene C_{14}
3. Eicosapentaenoic	20:5; 5,8,11,14,17-penta-ene C_{20}
4. γ-Linolenic	18:3; 6,9,12-tri-ene C_{18}
5. Docosahexaenoic	22:6; 4,7,10,13,16,19-hexa-ene C_{22}
6. Myristic	14:0; C_{14}
7. Arachidonic	20:4; 5,8,11,14-tetra-ene C_{20}
8. Linoleic	18:2; 9,12-di-ene C_{18}
9. Palmitoleic	16:1; 9-ene C_{16}
10. Homo-γ-linolenic	20:3;8,11,14-tri-ene C_{20}
11. Palmitic	16:0; C_{16}
12. Oleic	18:1; 9-ene C_{18}

[a] Chain-length and number of olefin groups in the molecule; e.g. 14:1 refers to a C_{14} fatty acid with one olefin group.

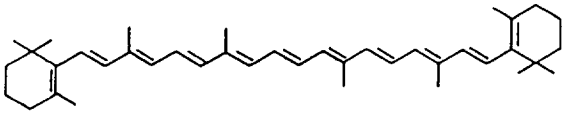
2.4. Treatment of experimental data

Parts II and III [1,16] describe the derivation of experimental values of δS and $\delta(\Delta t_R)$; see the examples of Fig. 1 and 2 of Part III [1]. In the latter examples, a straight-line best-fit is used to determine values of δS and $\delta(\Delta t_R)$, and a similar procedure was used for most (but not all) of the other systems studied in Part III [1] and the present paper. When the scatter of data points about any best-fit curve is large, there is often little difference in the standard deviations calculated for linear vs. non-linear curves. In some cases encountered in the present study, it was clear that a straight-line fit was inappropriate, and in these cases a two-segment linear fit was used instead. This approach would work reasonably well, for example, in the extreme examples cited in Fig. 5 of Part II [1] for a "regular" sample. It can be argued that best-fit curves that are restricted to linear or even two-segment cases still introduce some error into resulting values of δS and $\delta(\Delta t_R)$ and derived values of $\Delta\log \alpha^*$. This is correct, but we believe that these errors are small when compared to other sources of uncertainty, not the least being the limited number of compounds contained in any one sample.

3. Results and discussion

Selectivity data as a function of temperature and gradient steepness were obtained for the preceding non-ionic samples and supplemented with information from the literature and Part III [1]. The following discussion is organized by sample type.

Table 4
Components of some of the carotenoid sample (laboratory G). These compounds are derivatives of carotene, with substitution by various polar groups as indicated below



β-Carotene

Compound	Number of polar substituents				Total
	–OH	Ester[a]	Epoxy	Keto	
Peridinin	2	2	1		5
19′-Butanoyloxyfucoxanthin	2	1	1	1	5
Fucoxanthin[b]	2	1	1	1	5
19′-Hexanoyloxyfucoxanthin	2	1	1	1	5
Neoxanthin	3		1	1	5
Prasinoxanthin	3			1	4
Diadinoxanthin	2			1	3
Alloxanthin	2				2
Diatoxanthin	2				2
Lutein	2				2
Zeaxanthin	2				2

[a] Ester or actone group.
[b] Three isomers in the sample.

3.1. Hydrophobic, strongly-retained samples

The PAH, fatty acid ester, and carotenoid samples reported here are all relatively nonpolar and require large %B-values for elution with reasonable *k*-values.

3.1.1. Polycyclic aromatic hydrocarbons

Table 5 and Table 6 summarize *T*- and *b*-selectivity values ($\Delta\log\alpha^*$) for the PAH sample with the two columns (monomeric, polymeric column-1) used by laboratory E. Additional data from the literature [17] are listed for "polymeric column-2". *T*-selectivity is marginally greater for the polymeric-1 (0.03) vs. monomeric (0.01) column, but is not very pronounced in either case. All three columns exhibit greater *b*-selectivity (0.04–0.08). Useful changes in band-spacing have been reported for the PAHs as a result of changes in gradient steepness or %B [17,18] and temperature [4,19], despite these relatively small values of $\Delta\log\alpha^*$ (corresponding to maximum average changes of α of 7% [*T*] and 20% [*b*]). That is, changes in α that are >5% can be quite significant in HPLC method development.

3.1.2. Fatty acid methyl esters

These compounds have a chain-length of 12 to 22 carbons, with variable unsaturation and an occasional polar substituent in addition to the methylester group. In some respects, they resemble a homologous series, for which *b*- and *T*-selectivity should be negligible. The fatty acid ester sample of Table 2 (mainly mono-unsaturated) shows negligible *T*-selectivity (0.01 for either MeOH or ACN as B-solvent) in separations with the monomeric column. This is further illustrated in Fig. 1a, where Δt_R for a change in temperature (70 to 30°C) is plotted vs. t_R. For the polymeric column, *T*-selectivity is considerably greater ($\Delta\log\alpha^*=0.07$). However, this is due mainly to the anomalously large Δt_R-values for the saturated compounds. This is illustrated in Fig. 1b, where the dark circles refer to saturated fatty acid esters (9 and 14 of Table 2). At higher temperatures (70°C), the retention of this sample is similar on both mono-

Table 5
Summary of temperature-selectivity as a function of sample type and pH (nonionized samples)

Sample	n[a]	T[b] (°C)	t_G^c (min)	$\Delta\log\alpha^*$ (T)		
				Low t_G	High t_G	Average[d]
Polycyclic aromatic hydrocarbons						
Monomeric column[e]	10	25, 45	30, 90	0.01	0.01	0.01
Polymeric column-1[e]	8	25, 45	30, 90	0.04	0.02	0.03
Polymeric column-2[f]	15	35	30, 90			
Fatty acid methyl esters						
Monomeric column[g]						
MeOH	14	30, 70	30, 90	0.02	0.01	0.01
ACN	14	30, 70	30, 90	0.02	0.01	0.01
Polymeric column[g]						
ACN	14	40, 70	30, 90		0.07	0.07
Literature data[h]						
ACN	12	40, 70	isocratic			0.01
Carotenoids[i]						
Mobile phase-1	13	40, 60	25, 75	0.13	0.08	0.10
Mobile phase-2	13	45, 55	25, 75	0.16	0.17	0.17
Pharmaceuticals[j]	9	35, 75	30, 90	0.04	0.02	0.03
Nitro compounds[k]	18	35, 45	31			0.06
Nonbasic drugs[l]	20	30, 66.3	20, 60	0.11	0.09	0.10
Benzoic acids (pH 2.6)[m]	8	24.6, 69.7	15, 45	0.06	0.07	0.07
Anilines (pH 5.6)[m]	9	25.5, 69.7	1,3% B/min	0.08	0.09	0.09
Average					0.06	

[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 E.
[f] Data of Ref. [7].
[g] Data from laboratory F.
[h] Data of Ref. [12].
[i] Data from laboratory G.
[j] Data from laboratory D.
[k] Data from Ref. [8,22].
[l] Data from laboratory B.
[m] Repeated data from Table 3, Part II [1].

meric and polymeric columns (Fig. 1c), but at lower temperatures the saturated esters are preferentially retained on the polymeric column (Fig. 1d).

The foregoing behavior of the fatty acid esters and the polymeric column is similar to that of the PAHs with monomeric vs. polymeric columns and may reflect differences in shape of the fatty acid ester sample. *Cis*-unsaturation will cause a "bend" in the molecule, suggesting that shape selectivity may be involved in the T-selectivities of both the PAHs and fatty acid esters. The sample of Table 2 exhibits small and comparable b-selectivity values ($\Delta\log\alpha^*=0.02$–0.04) for either methanol or acetonitrile as B-solvent and for either monomeric or polymeric columns (Table 6).

The fatty acid ester sample of Table 3 differs from that of Table 2 in having a wider range of unsaturation (0–6 olefinic groups). This sample and column exhibit minimal T-selectivity ($\Delta\log\alpha^*=0.01$) and in this respect resemble the monomeric column and

Table 6
Summary of gradient–steepness selectivity as a function of sample type and pH (non-ionized samples)

Sample	$\Delta\log\alpha^*$ (b)			r^2
	Low T	High T	Average[d]	
Polycyclic aromatic hydrocarbons				
Monomeric column[e]	0.04	0.04	0.04	–
Polymeric column-1[e]	0.08	0.08	0.08	–
Polymeric column-2[f]			0.04	
Fatty acid methyl esters				
Monomeric column[g]				
MeOH	0.04	0.04	0.04	–
ACN	0.02	0.03	0.02	–
Polymeric column[g]				
ACN	0.03	0.03	0.03	–
Literature data[h]				
ACN			0.11	0.19
Carotenoids[i]				
Mobile phase-2	–	0.05	0.05	–
Mobile phase-2	0.03	0.03	0.03	–
Pharmaceuticals	0.17	0.16	0.17	–
Nitro compounds[k]			0.07	–
Nonbasic drugs[l]	0.24	0.25	0.24	0.40
Benzoic acids (pH 2.6)[m]			0.21	
Anilines (pH 5.6)[m]			0.12	
Average			0.09	

Same samples and conditions as Table 5. The correlation coefficient r^2 for the dependence of Δt_R on S is also given.
[a–k] see Table 3, except [d]: average of low and high T values, arbitrarily rounded to favor the low t_G value.

sample of laboratory F. However, b-selectivity is much more pronounced (0.11). Values of S at 50°C are plotted vs. k (90% ACN) for this column in Fig. 2 (the plot of Fig. 2 for isocratic data is equivalent to earlier plots of S vs. t_R for gradient data). Relative to the solid curve through the data points for the saturated compounds ("0-ene"), data for compounds with increasing unsaturation ("1-ene", "2-ene", etc.) lie on successively higher curves. Each olefin group in the molecule appears to increase S, relative to a more saturated compound with similar retention (i.e. smaller carbon number). Thus, an increase in b or %B is expected to decrease retention for more unsaturated compounds relative to less saturated compounds. Similar, but less pronounced trends in b-selectivity were observed for the sample of Table 2 (which lacks the wide range of unsaturation found in the sample of Table 3).

3.1.3. Carotenoids

The 13 carotenoids of Table 4 consist of a C_{38} hydrocarbon nucleus (carotenoid) substituted by varying numbers of oxygen-containing functional groups. These compounds therefore resemble the fatty acid esters in terms of being relatively nonpolar, but the extent of non-hydrocarbon substitution is greater and more variable. A special C_{30} column [20] was used with two different gradient times (25 and 75 min) and two different: mobile phases: 1 and 2 (see Section 2).

Temperatures of 40 and 60°C were used with mobile phase 1, and 45 and 55°C with mobile phase 2. As seen in Table 5 and Table 6, b- and T-selectivities are on average greater for this sample compared to the PAH and fatty acid ester samples: $\Delta\log\alpha^*$=0.08–0.17 (T) and 0.03–0.05 (b). This sample is also unusual in that T-selectivity is about three-fold more important than b-selectivity. This may be related to conformational changes in the stationary phase with temperature [21], which are expected to be especially pronounced in this temperature range for this very-long-chain bonded phase.

3.1.4. Summary

For this group of relatively hydrophobic, strongly retained samples, T-selectivity is not very significant ($\Delta\log\alpha^*<0.04$), except for the separation of long-chain compounds on the polymeric or C_{30} column: fatty acid methyl esters ($\Delta\log\alpha^*$ ($[T]$=0.07) and carotenoids (0.10–0.17). For separations of the same PAH or fatty acid ester sample, the polymeric column gave slightly greater values of $\Delta\log\alpha^*(b)$. The present data suggest that a polymeric or very-long-chain bonded-phase column ($\gg C_{18}$) is preferred for the separation of these and similar samples. Except for the carotenoids, b-selectivity was generally greater than T-selectivity.

3.2. Other samples

3.2.1. Pharmaceuticals

These neutral compounds are characterized by limited polar-group substitution onto a C_8–C_{30} hy-

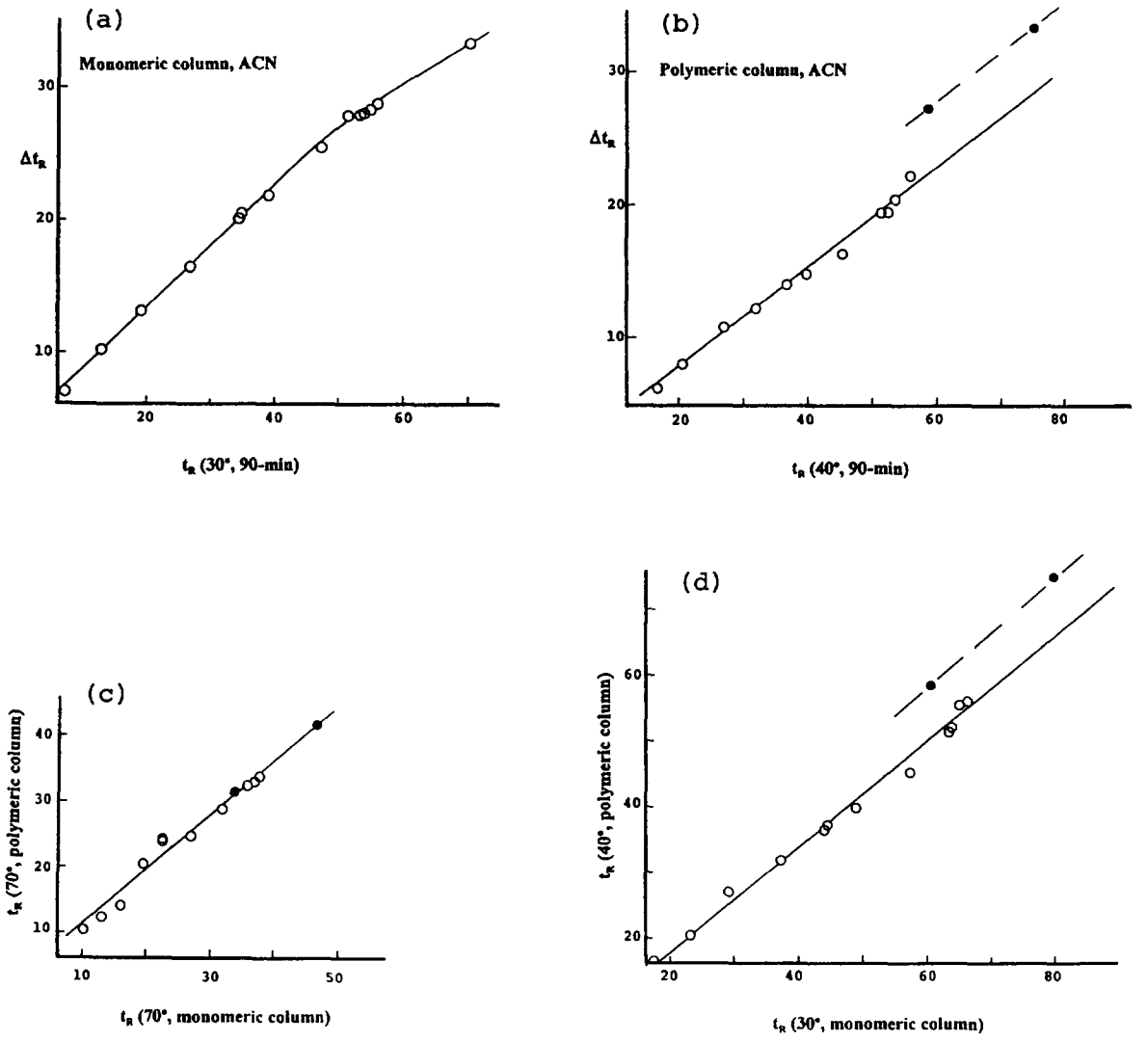


Fig. 1. Temperature selectivity effects for the fatty acid methyl ester sample of laboratory F. (a) Δt_R (30°C vs. 70°C) vs. t_R (30°C) for a 90-min gradient and monomeric column; (b) Δt_R (40°C vs. 70°C) vs. t_R (40°C) for a 90-min gradient and polymeric column; (c) comparison of retention times for polymeric vs. monomeric columns at high temperature (70°C); (d) comparison of retention times for polymeric vs. monomeric columns at low temperature (30 or 40°C). (○) Unsaturated fatty acid esters; (●) saturated esters [not indicated in (a)].

drocarbon residue (Table 5 of Part I [14]). Temperature-selectivity for this sample is small ($\Delta\log \alpha^*=0.03$), but *b*-selectivity is very significant (0.17).

3.2.2. Nitroaromatics

Several studies have been reported [7,8,22] for the isocratic or gradient separation of nitro-substituted aromatics as a function of (a) %B or gradient steepness and/or (b) temperature. One study [22], using a sample that contained benzene, toluene and xylene substituted by zero to two nitro groups, yields a value of $\Delta\log \alpha^*=0.07$ for %B-selectivity (equivalent to *b*-selectivity). As a result of this significant *b*-selectivity, it was possible to separate this sample simply by varying mobile phase %MeOH.

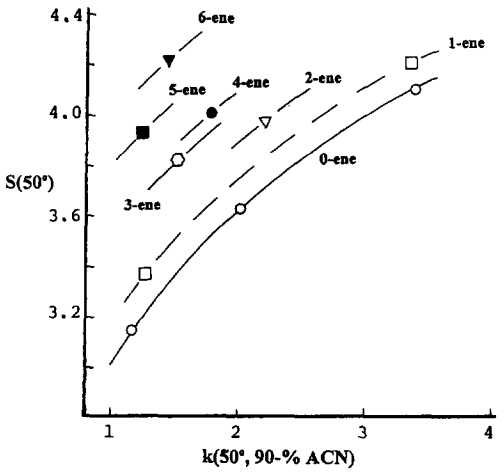


Fig. 2. Dependence of S on solute retention for fatty acid esters from study of Ref. [11]. Values of S (50°C) are average values of S at 50°C; values of k are for 50°C and 90% ACN–water mobile phase. Data-points are characterized by number of olefin groups in the molecule; e.g. 0-ene refers to saturated compounds, 1-ene to mono-unsaturated compounds, etc.

Several nitroaromatics from the study of [7] were remeasured [8] under conditions that should provide more reliable values for t_R as a function of temperature. After rejecting data for early (less reliable) bands and a few statistically-defined outliers, a value of $\Delta\log\alpha^*(T)\approx 0.06$ for a change in temperature was calculated. It appears that b- and T-selectivities are comparable and significant for these nitroaromatic samples.

3.2.3. Non-basic drugs

The drug sample from laboratory B contains 21 compounds that are either acidic or neutral; these non-basic drugs are expected to be uncharged under the conditions for these separations (pH 2). Selectivity values for this sample are shown in Table 5 and Table 6: $\Delta\log\alpha^*=0.10$ (T) and 0.24 (b).

3.2.4. Non-ionized benzoic acids anilines

These data were discussed in Part III [1] and are repeated in Table 5 and Table 6 for comparison with the other neutral compounds reported here. Values of $\Delta\log\alpha^*(T)=0.07$–$0.09$ are similar to those for the non-basic drug sample (0.09), while values of $\Delta\log\alpha^*(b)$ are more variable (0.12–0.24) for these two samples.

3.3. Significance of values of $\Delta\log\alpha^*$ reported in this study

The values of $\Delta\log\alpha^*$ reported in Table 5 and Table 6 and in Part III [1] for these samples and conditions are believed to be reproducible within ± 0.01–0.02 units (see Discussion of Part III [1] and note the accuracy of predictions of α in Part I [14]). A more important question is whether general conclusions can be drawn from studies based on a relatively small number of compounds (compared to the large organic-compound universe). One concern is that a few atypical compounds in these samples might seriously distort resulting values of $\Delta\log\alpha^*$. Where the number n of compounds in the sample is small, this is a valid concern. We have tested for this possibility below, by examining whether values of δS and $\delta(\Delta t_R)$ from the present study are normally distributed.

Another measure of the reliability of our conclusions is whether they seem consistent and reasonable in terms of trends of selectivity with structure and conditions. For this, the reader will have to compare Section 4 with the $\Delta\log\alpha^*$ data of this and the preceding paper. A more detailed investigation of selectivity vs. structure will be presented [23], as part of a more general examination of selectivity vs. conditions (T, b, solvent type, column type) and sample composition.

3.3.1. Random distribution of δS and $\delta(\Delta t_R)$ values

A reviewer of this paper has made two interesting observations. First, when a change in temperature or gradient steepness is made, typically only a few bands in the chromatogram appear to exhibit significant changes in selectivity. Second, several of the plots of S or Δt_R vs. t_R show most of the data points lying close to the best-fit curve, with a few points deviating much further from the curve (e.g. Fig. 3.2 and 3.3 of Part III [1]). These two generalizations suggest that b- and T-selectivity effects may not be normally distributed. Rather, only a few bands in the chromatogram may be markedly affected by a change in gradient steepness or temperature, so that resulting values of $\Delta\log\alpha^*$ (1 S.D.) for the entire sample are misleading. That is, these (rather large) values of $\Delta\log\alpha^*$ for most samples suggest that a

Table 7
Cumulative frequency distribution of values of δS and $\delta(\Delta t_R)$ for samples from the present study (Part III and IV). Cumulative frequency distributions vs. a Gaussian distribution

σ	Frequency distributions		
	Gaussian	δS	$\delta(t_R)$
<-2	2	3	2
-2 to -1.5	7	7	5
-1.5 to -1.0	16	16	13
-1.0 to -0.5	31	31	30
-0.5 to 0.0	50	50	53
0.0 to 0.5	69	70	73
0.5 to 1.0	84	85	87
1.0 to 1.5	93	93	94
1.5 to 2.0	98	97	98

change in temperature or gradient steepness will in most cases result in a separation of previously overlapping bands, whereas this actually may be true for only a small fraction of the sample components.

We tested this conclusion as follows. Values of δS and $\delta(\Delta t_R)$ for each sample were first normalized by dividing these values by the standard deviation of δS or $\delta(\Delta t_R)$ for each sample. Resulting values of δS or $\delta(\Delta t_R)$ (now in units of σ [S.D.]) were next combined and grouped as follows: $\langle -2\sigma$, -2 to -1.5σ, -1.5 to -1.0σ, etc.). These latter values were then summed to give a cumulative distribution of δS or $\delta(\Delta t_R)$ vs. σ. The latter distribution was then compared with a Gaussian distribution, as summarized in Table 7.

The comparison of Table 7 indicates that within experimental error these values of δS and $\delta(\Delta t_R)$ are normally distributed. Therefore, values of $\Delta\log \alpha^*$ summarized here and in Part III [1] seem to be reliable indicators of the value of a change in b or T for the purpose of changing selectivity. The common observation that the selectivity of only one or two pairs of bands in a chromatogram are affected by a change in b or T is likely due to the fact that similar changes in α for more widely separated band-pairs are generally less obvious.

4. Conclusions

For the nine samples in the present study, the average values of temperature and gradient–steepness selectivities were $\Delta\log \alpha^*$ equal 0.06 (T) and 0.09 (b). These values can be compared with corresponding values for the ionized samples of Part III [1]: 0.18 (T) and 0.24 (b). It appears that b- and T-selectivities are about three-fold more important for ionized vs. un-ionized samples, but this generalization often fails for specific samples. For both sample types, b-selectivity is about 1.5 times as important as T-selectivity. However, even for the case of non-ionized samples and the use of temperature to control band spacing, changes in α that are large enough to resolve two previously overlapped bands seem likely. See Ref. [23] for a further discussion.

Changes in α as a result of varying b or T are generally independent of each other (little correlation of values of S or B), suggesting that the combined variation of temperature and gradient. steepness (or %B) should be an effective first step in HPLC method development. This approach seems more favored for ionized vs. non-ionized samples, but good results can be expected for both sample types.

Hydrophobic samples with little or no substitution of the molecules by polar functional groups are the least promising candidates for b- and T-optimization. However, past examples from the literature have demonstrated that separations of even these sample types can often be improved by changes in temperature or gradient steepness. Available evidence suggests that the use of long-chain (e.g. C_{30}) or polymeric bonded-phase columns provides some increase in b- and T-selectivities for hydrophobic samples, compared to the use of the more common monomeric-silane silica based packings.

While a total of 15 ionized or neutral samples (more than 250 solutes and about 1000 experiments) were investigated in the present study (Parts III and IV), it is almost certain that some samples will contain similar, overlapping compounds which exhibit b- and T-selectivities that are so small as not to be useful for HPLC method development. Such samples will require a different method development approach (as in Ref. [24]) or the use of special conditions for individual sample types (e.g. isomers).

Another impediment to the variation of temperature in HPLC method development is that about half of all HPLC systems are not equipped with column thermostating means. However, there are other

reasons to encourage the control of column temperature.

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