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Column selectivity in reversed-phase liquid chromatography II. Effect of a change in conditions

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Abstract

The isocratic retention of 67 widely-different solutes in reversed-phase liquid chromatography (RP-LC) has been investigated as a function of temperature and mobile phase composition (% *B*) for three different C_{18} columns. Similar studies were also carried out in a gradient mode, where temperature, gradient time and solvent type were varied. These results show that changes in retention with these conditions are similar for each of these three columns. This suggests that relative column selectivity as defined by experiments for one set of experimental conditions will be approximately applicable for other conditions, with the exception of changes in mobile phase pH—which can affect values of the column parameter ***C*** (a measure of silanol ionization). Column selectivity as a function of pH was explored for several columns.

Keywords: Temperature effects; Mobile phase composition; Retention prediction; Column selectivity; Selectivity; Stationary phase, LC

1. Introduction

The preceding paper ([1], Part I) presented retention data in support of an empirical equation that describes RP-LC column selectivity:

$$\log \alpha \equiv \log(k/k_{ref}) = \eta' \boldsymbol{H} + \sigma' \boldsymbol{S} + \beta' \boldsymbol{A} + \alpha' \boldsymbol{B} + \kappa' \boldsymbol{C} \quad (1)$$

The retention factor k of a given solute for specified separation conditions (only the column varying) is related to properties of the solute molecule (η', σ', β', α' and κ') and the column (***H***, ***S***, ***A***, ***B*** and ***C***). The quantity k_{ref} refers to the value of k for the solute ethylbenzene. Eq. (1) was shown to be applicable for a wide range in solute structures and a narrower range in RP-LC columns (monomeric C_8 and C_{18} phases without an embedded polar group [1]). For experiments reported in Part I [1], column temperature and mobile phase composition were held constant. In the present study, the effect of a change in these conditions on the applicability of Eq. (1) is examined with two questions in mind. First, when temperature or the mobile phase is varied, is the primary consequence a change in the solute parameters (η', σ', etc.) or the column parameters (***H***, ***S***, etc.)? If the column parameters are relatively insensitive to changes in conditions, then column selectivity

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is adequately characterized by measurements of retention under a single set of conditions. Second, can changes in retention with conditions provide further insight into the origin of the various terms of Eq. (1)? The first question (column and solute parameters vs. conditions) will be addressed in the present paper. The second question (the physicochemical basis of each term in Eq. (1)), is deferred to Part III [2].

2. Theory

As suggested by previous reports (see immediately below) and further supported by data from the present paper (Results and discussion), it appears that the solute parameters of Eq. (1) are affected by a change in separation conditions, but column parameters (and column selectivity) are much less dependent on conditions—except for mobile phase pH. This means that measurements of column selectivity (values of ***H***, ***S***, etc.) for one set of conditions will be approximately valid for other conditions, hence simplifying the routine characterization of column selectivity.

2.1. Column parameters vs. conditions

When either temperature or mobile phase composition is varied, k can change. If this change in $\log k$ ($\delta \log k$) as a result of change in some condition ''X'' ($\delta \log k$[X]) has the same value for any *individual* solute and *different* columns, it then follows that the column parameters of Eq. (1) will be unaffected by that particular change in conditions. The latter conclusion (an absence of change in column selectivity for a change in separation conditions) can be demonstrated as follows. Column selectivity can be defined as the change in separation factors α for a given solute band-pair i and columns 1 and 2: $\log \alpha_{i2} - \log \alpha_{i1}$. If values of $\delta \log k(X)$ for a solute are the same for different columns 1 and 2, then a change in the condition X will also result in a change in $\log \alpha_i$ ($\delta \log \alpha_i$) that is the same for the two columns. Column selectivity for a given band-pair i and the new conditions is then $(\log \alpha_{i2} + \delta \log \alpha_i) - (\log \alpha_{i1} + \delta \log \alpha_i)$, which is the same as prior to the change in conditions (for which $\delta \log \alpha_i = 0$). If there is no change in column selectivity, there should be no change in the column selectivity parameters ***H***, ***S***, etc. An example of this was noted in Part I [1], where a change in temperature by 10 °C was found to have a much larger effect on values of the solute parameters (η', -0.008 ± 0.023 [1 SD]; σ', -0.023 ± 0.074; β', -0.001 ± 0.001), compared to changes in the column parameters (***H***, 0.000 ± 0.003; ***S***, 0.003 ± 0.009; ***A***, 0.000 ± 0.009).

We further tested this premise (no change in ***H***, ***S***, etc. with conditions, when $\delta \log \alpha$ is the same for different columns) in the following way. Table 1 provides experimental values of $\delta \log k$ for a particular column as a result of various changes in conditions. We calculated values of $\log k$ for different conditions and different columns by simply adding values of $\delta \log k$ from Table 1 to values of $\log k$ for different solutes and columns for the starting condition (values of Table 3 in Part I [1]). The resulting values of $\log k$ for a change in conditions thus assumed that $\delta \log k$ (and therefore $\delta \log \alpha$) was constant for each column ≠1–10 in Part I [1]. Values of $\log k$ for this change in conditions could then be used as in step ≠7 in Table 4 of Part I [1] to calculate values of η', σ', etc., using the same values of ***H***, ***S***, etc. as derived in Table 5 of Part I [1]. The resulting agreement of experimental and calculated values of $\log \alpha$ was found to be exactly the same (± 0.004, 1 SD) as in the original correlation prior to a change in conditions [1]. Poorer agreement was found when it was assumed that solute parameters do not change with conditions (and column parameters can).

A reviewer has questioned some aspects of this conclusion, as discussed in Appendix A. Thus, more detailed analysis of Eq.1 suggests that if values of $\delta \log k$ are equal for a given solute and different columns, then values of the solute parameters must remain constant, while the column parameters change. Our above results draw the opposite conclusion, but it turns out that either hypothesis leads to the same result: columns of similar composition do not change their *relative* selectivity properties for a small to moderate change in conditions, and therefore the measurement of values of ***H***, ***S***, etc. for one set of conditions provides a reasonable measure of column selectivity for other conditions (expect a change in pH). See Appendix A for further details.

Table 1
Change in log k for various solutes as a result of change in temperature or mobile phase composition (only one variable changed at a time)

Solute	Change in log k (δ log k) for change in various conditions				
	+10 °C	−10% ACN	MeOH[a]	THF[a]	MTBE[a]
1. Benzene	−0.049	0.262	−0.016	−0.068	−0.014
2. Toluene	−0.053	0.308	−0.007	−0.079	−0.035
3. Ethylbenzene	−0.056	0.359	0.000	−0.090	−0.054
4. *p*-Xylene	−0.060	0.358	0.002	−0.094	−0.059
5. Propylbenzene	−0.065	0.412	0.010	−0.106	−0.078
6. Butylbenzene	−0.069	0.462	0.017	−0.119	−0.103
7. Naphthalene	−0.063	0.366	0.003	−0.101	−0.078
8. 4-Chlorotoluene	−0.059	0.404	0.003	−0.089	−0.054
9. *p*-Dichlorobenzene	−0.061	0.371	0.002	−0.085	−0.046
10. Benzotrichloride	−0.060	0.426	0.011	−0.098	−0.079
11. Bromobenzene	−0.056	0.325	−0.005	−0.084	−0.044
12. 1-Nitropropane	−0.049	0.194	−0.038	−0.070	−0.017
13. Nitrobenzene	−0.054	0.262	−0.020	−0.077	−0.041
14. 4-Nitrotoluene	−0.056	0.310	−0.009	−0.090	−0.069
15. 4-Nitrobenzyl chloride	−0.061	0.338	−0.011	−0.089	−0.072
16. *N*-benzyl-formamide	−0.016	0.190	−0.011	−0.063	−0.053
17. Anisole	−0.055	0.267	−0.015	−0.077	−0.041
18. Benzyl alcohol	−0.029	0.177	−0.017	−0.035	0.001
19. 3-Phenyl propanol	−0.027	0.257	0.006	−0.054	−0.035
20. 5-Phenyl pentanol	−0.071	0.345	0.027	−0.074	−0.085
21. Phenol	−0.049	0.200	−0.023	−0.012	0.049
22. *p*-Chlorophenol	−0.048	0.274	−0.002	−0.015	0.037
23. 2,3-Dihydroxy-napthalene	−0.048	0.279	0.001	−0.001	0.028
24. 1,3-Dihydroxy napthalene	−0.039	0.183	−0.038	−0.093	−0.068
25. Eugenol	−0.041	0.315	0.002	−0.060	−0.054
26. Danthron	−0.078	0.375	0.025	−0.115	−0.158
27. *n*-Propyl formate	−0.034	0.174	−0.030	−0.073	−0.022
28. Methylbenzoate	−0.055	0.267	−0.012	−0.088	−0.064
29. Benzonitrile	−0.052	0.235	−0.030	−0.080	−0.039
30. Coumarin	−0.045	0.267	−0.026	−0.082	−0.065
31. Acetophenone	−0.041	0.226	−0.021	−0.082	−0.056
32. Benzophenone	−0.057	0.364	0.001	−0.115	−0.120
33. *cis*-Chalcone	−0.059	0.421	0.006	−0.126	−0.145
34. *trans*-Chalcone	−0.065	0.439	0.015	−0.132	−0.163
35. *cis*-4-Nitro-chalcone	−0.067	0.440	0.009	−0.122	−0.148
36. *trans*-4-Nitro-chalcone	−0.080	0.438	0.019	−0.130	−0.176
37. *cis*-4-Methoxy-chalcone	−0.059	0.429	0.011	−0.134	−0.167
38. *trans*-4-Methoxy-chalcone	−0.066	0.456	0.021	−0.144	−0.191
39. Prednisone	−0.015	0.298	0.046	−0.062	−0.127
40. Hydrocortisone	−0.003	0.296	0.064	−0.034	−0.117
41. Mephenytoin	−0.033	0.269	0.004	−0.050	−0.018
42. Oxazepam	−0.019	0.329	0.058	−0.034	−0.106
43. Flunitrazepam	−0.034	0.347	0.015	−0.097	−0.131
44. 5,5-Diphenyl-hydantoin	0.016	0.281	0.036	−0.020	−0.003
45. *N*,*N*-dimethylacetamide	0.045	0.073	−0.037	−0.191	−0.147
46. Amitriptyline	−0.007	0.347	0.146	−0.082	−0.234
47. Diphenhydramine	−0.007	0.350	0.126	−0.082	−0.200
48. Propranolol	0.009	0.420	0.157	0.001	−0.129
49. Nortriptyline	−0.004	0.350	0.167	−0.040	−0.201
50. Prolintane	−0.002	0.332	0.077	−0.089	−0.153
51. 4-*n*-Pentylaniline	0.066	0.172	0.070	−0.023	−0.094
52. 4-*n*-Hexylaniline	0.059	0.221	0.084	−0.033	−0.118

Table 1. Continued

Solute	Change in log k (δ log k) for change in various conditions				
	+10 °C	−10% ACN	MeOH[a]	THF[a]	MTBE[a]
53. 4-*n*-Heptylaniline	0.051	0.267	0.092	−0.045	−0.144
54. *N*-ethylaniline	0.091	0.323	0.003	0.013	−0.014
55. 2-Phenyl pyridine	0.012	0.122	0.028	−0.038	−0.066
56. Diclofenac acid	−0.057	0.462	0.103	0.007	−0.057
57. Mefenamic acid	−0.059	0.492	0.114	0.010	−0.048
58. Ketoprofen	−0.044	0.386	0.077	−0.006	−0.062
59. Diflunisal	−0.111	0.422	0.045	−0.018	0.003
60. 4-*n*-Butylbenzoic acid	−0.048	0.417	0.097	0.007	−0.030
61. 4-*n*-Pentylbenzoic acid	−0.051	0.463	0.115	0.001	−0.053
62. 4-*n*-Hexylbenzoic acid	−0.056	0.512	0.132	−0.009	−0.079
63. 3-Cyanobenzoic acid	−0.067	0.275	0.006	0.017	0.059
64. 2-Nitrobenzoic acid	−0.088	0.219	−0.037	0.004	0.068
65. 3-Nitrobenzoic acid	−0.069	0.252	0.020	0.024	0.058
66. 2,6-Dimethylbenzoic acid	−0.042	0.343	0.026	0.032	0.056
67. 2-Fluorobenzoic acid	−0.050	0.121	0.007	0.015	0.061

For original (unchanged) isocratic conditions and other details, see Section 3 (neutral solutes ≠1–45 measured on Symmetry column with ACN–water as mobile phase; ionic solutes ≠46–67 measured on SB-100 column with ACN buffer). In every case, the buffer concentration of the final mobile phase was held constant at 31.2 m*M*.

[a] 10% of indicated solvent replaces 10% ACN in B-solvent, e.g. 50% ACN–buffer is replaced by 45% ACN–5% MeOH–50% buffer, etc.

2.2. Change in solvent strength (% B)

Changes in retention where only % B is varied can be approximated by Eq. (2):

$$\log k = \log k_w - S\phi \quad (2)$$

Here, k_w refers to the extrapolated value of k for 0% B ($\phi = 0$), and S (not to be confused with the column parameter $\boldsymbol{S}$) varies with the solute. A change in retention ($\delta \log k$[% B]) with change in ϕ ($\delta\phi$) is then given as:

$$\delta \log k(\%\, B) = -S\delta\phi \quad (3)$$

If a value of S for any given solute does not change for different columns, the quantity $\delta \log k$ for a given solute and some change in % B will be the same for all columns, and values of $\boldsymbol{H}$, $\boldsymbol{S}$, etc. for a given column will therefore not vary with ϕ. Several studies suggest that values of S are indeed approximately independent of differences in the column [4–7]. Gradient data also allow the measurement of values of $S = d(\log k)/\mathrm{d}\phi$ (Eq. (3)), as discussed in Ref. [7].

2.3. Changes in temperature

Isocratic retention as a function of temperature can be described generally by the equation:

$$\log k = A + B/T_K \quad (4)$$

Here, A and B are constants for a given solute, and T_K refers to temperature in K. A change in retention ($\delta \log k$[T]) with change in T from T_1 to T_2 is then given as:

$$\delta \log k[\mathrm{T}] = B([1/\mathrm{T}_2] - [1/\mathrm{T}_1]) \quad (4a)$$

In gradient elution [8], retention time t_R as a function of temperature is given approximately as:

$$t_R \approx A'' - B''T_K \quad (5)$$

A change in retention time (δt_R) with change in T from T_1 to T_2 is then:

$$\delta t_R \approx B''(T_1 - T_2) \quad (5a)$$

Two studies [7,9] suggest that values of B or B'' for different solutes are similar for different columns, so that relative column selectivity should not vary with T.

2.4. Changes in mobile phase pH

A change in mobile phase pH primarily affects the ionization of acidic or basic solutes, with predictable changes in retention based on the Henderson–Hasselbach equation [10]. Consequently, resulting values of $\delta \log k$(pH) for each solute should be the same,

regardless of the column used. As with changes in % ***B*** or *T* discussed above, the column parameters of Eq. (1) should therefore exhibit little change as mobile phase pH is varied. There is, however, an important exception to this conclusion: a secondary effect of a change in pH will be to alter the degree of ionization of silanol groups in the bonded phase, which will mainly affect the retention of protonated bases and values of the column parameter ***C***.

*2.4.1. Relation of **C** to mobile phase pH*

Assume a cationic solute X^+, corresponding either to a fully protonated base or a quaternary ammonium compound such as berberine (≠91) or bicuculline (≠92); see Fig. 1 of Part III [2] for structures. For the numbering below of other solutes, see Parts I [1] or III [2]. For a change in pH, Eq. (1) can be written as (first approximation):

$$\log k = c_x + \kappa' \boldsymbol{C} \quad (6)$$

where c_x is a constant for a given solute X^+. Values of κ' are similar for different completely ionized cationic solutes, e.g. $0.8 \leq \kappa' \leq 1.2$ for the five strong bases of Part I (solutes ≠46–50), or $\kappa' \approx 1$ for all univalent cationic solutes. Consequently, for all univalent cationic solutes that are in the fully-ionized form:

$$\log k \approx c_x + \boldsymbol{C} \quad (7)$$

The verification of Eq. (7) and its application to measurements of ***C*** as a function of pH are examined in Results and discussion.

2.5. Changes in ion-pairing

A simplified picture of ion-pair chromatography (IPC, [11]) suggests that molecules of the IPC reagent cover part of the bonded phase surface, resulting in ion-exchange retention by absorbed reagent and RP-LC retention by the uncovered bonded phase. The extent of surface coverage by reagent thus depends on the reagent concentration in the mobile phase. When the reagent concentration is adjusted to give the same fractional coverage of the bonded-phase surface, changes in retention ($\delta \log k$) with IPC reagent concentration should be similar for different columns. This suggests that the column parameters of Eq. (1) should be relatively unaffected by changes in ion-pairing, when similar changes in reagent concentration are made.

2.6. Changes in solvent

The effect on column selectivity of a change in mobile-phase organic solvent (acetonitrile, methanol, tetrahydrofuran, etc.) has received little prior attention in the literature; i.e. we know of no comparisons of changes in *k* as a result of change in solvent *for different columns*. Similarly, there is no theoretical basis by which to predict how the column parameters of Eq. (1) might vary with a change in solvent. Data presented here and discussed in a later section serve to clarify this question.

3. Experimental

3.1. Equipment, materials and procedures

These are essentially the same as in Ref. [1], except where indicated otherwise or as noted in table below:

Table in which data are reported	Separation conditions (isocratic experiments)
Table 1	Neutral solutes ≠1–45: 50% ACN–water Ionizable solutes ≠46–67: 50% ACN–buffer (B/A); buffer concentration in final mobile phase is 31.2 m*M* potassium phosphate, pH 2.8; indicated changes are for total mobile phase (solvents-A and -B combined)
Table 2	40 or 50% ACN–water
Table 8	Same as in Table 1, except for one-at-a-time changes indicated in the table
Fig. 6	50% ACN–buffer, buffer is 60 m*M* sodium citrate adjusted to given pH; 35 °C; 1.5 ml/min.

	Separation conditions (gradient experiments)	
	Solvent A	Solvent B
Table 3–5	5% ACN–buffer; buffer is 10 m*M* potassium phosphate, pH 2.8	81% ACN–buffer; buffer is 10 m*M* potassium phosphate, pH 2.8
Table 6	5% organic[a]–buffer; buffer is 10 m*M* potassium phosphate, pH 2.8	81% organic[a]–buffer; buffer is 10 m*M* potassium phosphate, pH 2.8

[a] Organic is either MeOH or THF, instead of ACN; i.e. a complete replacement of one organic by the other.

For short-hand designations of column type (e.g. SB-100, SB-90, etc.), see Table 2 of Ref. [1].
Solute numbering is given in Table 1. The system dwell volume was 2.03 ml.

3.2. Reproducibility of reported values of retention time t_R or k

Isocratic values of k reported here were determined in the same way as in Ref. [1] and were similarly repeatable ($\pm$0.4–0.5% in k), as measured by replicate values of k, or α-values for various homologous series. Gradient separations (5–81% B) were carried out with t_G equal 10 and 20 min and a temperature of either 35 or 50 °C. For each of these four conditions, six replicate injections were made of a system-suitability sample that consisted of 2-nitrobenzoic acid, amitriptyline, naphthalene, nitrobenzene and 4-*n*-butylbenzoic acid. The average standard deviation of retention times t_R for the system suitability sample was 0.011 min for t_G equal 10 min, and 0.017 for t_G equal 20 min. The corresponding uncertainty in values of k^* (comparable to uncertainty in isocratic values of k) can be estimated [12]: $\pm$0.6% ($t_G = 10$ min) and $\pm$0.4% ($t_G = 20$ min). These uncertainty values are similar to the uncertainty of isocratic values of k ($\pm$0.4–0.5%) reported in Part I [1], which is expected [12].

3.3. Calculation of values of $S = \mathrm{d}(\log[\mathrm{k}]/\mathrm{d}\phi$ and B''

Values of S were measured from isocratic and gradient data as described in Section 2. Values of B''/t_G were determined as described in Ref. [7]. It has been observed previously [7] and confirmed here that values of S from gradient data do not vary significantly with temperature, and values of B''/t_G from gradient data show little variation with t_G. Therefore, values of S (gradient data) reported here are the average of values at two temperatures (35 and 50 °C), and values of B''/t_G are the average of values for two different gradient times (10 and 20 min).

4. Results and discussion

Table 1 summarizes the effects of a change in various conditions on the retention of the 67 solutes studied in Part I [1]. These isocratic data were determined with the Symmetry column for the neutral solutes, and with the SB-100 column for the acidic and basic solutes. The data of Table 1 are reported as changes in $\log k$ ($\delta \log k$) for each solute and change of condition. Table 2 reports similar data for 27 neutral solutes, a change in % B (40 and 50% acetonitrile–buffer), and three different columns (Symmetry, SB-100 and SB-90). Tables 3–5 report gradient retention times for the same 67 solutes of Table 1 and three different columns (SB-100, SB-90 and Symmetry), for four different conditions both temperature T and gradient time t_G varying). Table 6 reports similar gradient data (38 solutes) for two columns (SB-100 and Symmetry) and a change in solvent (methanol [MeOH] or tetrahydrofuran [THF] replaces acetonitrile).

4.1. Effect on retention of a change in % B or gradient time, as a function of the column

The SB-90 and SB-100 columns of Table 2 are slightly different in selectivity, as measured by their column parameters, while the Symmetry and SB-100 columns are much more different (see data of Table 5 of Ref. [1]). Values of $S = \mathrm{d}(\log k)/\mathrm{d}\phi$ were calculated (Eq. (3)) for the neutral solutes and columns of Table 2, and the latter values are compared in Fig. 1 for (a) the S-90 vs. S-100 columns and (b) the Symmetry vs. S-100 columns. In each case, the best fit to the data is given as $y = Cx$ (where $1.00 \leq C \leq 1.01$), with a standard error (SE) of only 0.02–0.06 units; i.e. $\pm$0.6–1.8% differences in S between either of the two columns compared. For each of the three columns of Table 2, it appears that values of S for a given (neutral) solute are essentially the same, independent of the column; therefore, values of $\delta \log k$(% B) and relative column selectivity are unchanged for each column as % B is varied.

Tables 3–5 report retention data for the gradient separation of 67 neutral, acidic and basic solutes on the same three columns used in Table 2. Equivalent changes in selectivity can be achieved either by a change in isocratic % B or gradient time t_G [12]. The data of Tables 3–5 (which involve changes in t_G) were used to derive average values of S for each solute and column, as discussed in Section 3. In

Table 2
Isocratic retention of selected non-ionic solutes and three different columns: acetonitrile–water mobile phases, 35 °C; other conditions as in Section 3

Solute	log *k*					
	SB-100		SB-90		Symmetry	
	40% *B*	50% *B*	40% *B*	50% *B*	40% *B*	50% *B*
N-benzylformamide	−0.206	−0.439	−0.169	−0.406	−0.218	−0.466
Benzyl alcohol	−0.069	−0.279	−0.054	−0.268	−0.039	−0.255
Prednisone	0.081	−0.253	0.120	−0.213	−0.007	−0.359
Hydrocortisone	0.070	−0.253	0.104	−0.219	0.001	−0.343
Phenol	0.070	−0.162	0.077	−0.157	0.113	−0.121
1,3-Napthalenediol	0.146	−0.173	0.156	−0.164	0.184	−0.140
Coumarin	0.275	0.029	0.290	0.043	0.271	0.024
2,3-Napthalenediol	0.231	−0.076	0.239	−0.070	0.273	−0.039
1-Nitropropane	0.250	0.035	0.250	0.035	0.292	0.077
5,5-Diphenyldantoin	0.289	−0.069	0.301	−0.058	0.318	−0.045
Acetophenone	0.406	0.157	0.410	0.159	0.430	0.180
Benzonitrile	0.448	0.190	0.451	0.191	0.476	0.215
Nitrobenzene	0.607	0.322	0.605	0.320	0.644	0.359
Methyl benzoate	0.662	0.376	0.658	0.370	0.705	0.417
Eugenol	0.672	0.340	0.666	0.334	0.719	0.386
Anisole	0.713	0.426	0.700	0.414	0.777	0.489
Benzene	0.749	0.471	0.731	0.454	0.833	0.554
4-Nitrobenzylchloride	0.897	0.535	0.891	0.529	0.930	0.567
Toluene	1.016	0.693	0.989	0.666	1.107	0.781
Benzophenone	1.119	0.734	1.104	0.723	1.146	0.765
Bromobenzene	1.091	0.748	1.066	0.724	1.181	0.836
cis-4-Nitrochalcone	1.276	0.818	1.267	0.811	1.282	0.826
cis-4-Methoxychalcone	1.269	0.823	1.257	0.813	1.283	0.839
Naphthalene	1.235	0.856	1.209	0.829	1.322	0.939
p-Xylene	1.286	0.917	1.250	0.883	1.386	1.016
trans-4-Methoxychalcone	1.396	0.921	1.385	0.912	1.409	0.937
trans-4-Nitrochalcone	1.429	0.941	1.420	0.933	1.429	0.943

terms of changes in retention (k or k^*), the change in t_G (and k^*) by 2-fold in Tables 3–5 is equivalent to a change in isocratic % B by about 7%. Fig. 2a and b compare values of S for the SB-90 and Symmetry columns vs. values for the SB-100 column. In each case (Fig. 2a,b), there is a good correlation of values of S between columns (correlation coefficient $r =$ 0.97–0.99). However, values of S are seen to be lower on the SB-90 and Symmetry columns by 4 and 22%, respectively. This behavior contrasts with that observed in Fig. 1 for isocratic separation, but similar changes in S were noted in the gradient separations of [7], where values of S for different columns varied by as much as 25%. The latter difference in S values between columns for gradient vs. isocratic elution is not significant in terms of column selectivity; it can be explained in terms of the approximate nature of Eq. (3) and basic differences between isocratic and gradient elution (see Appendix B). Our conclusion from the data of Tables 2–4 is that relative selectivity does not change significantly with changes in either isocratic % B or gradient time t_G.

4.2. Effect on retention of a change in temperature as a function of the column

Fig. 3a and b show plots of the temperature coefficient of retention (B''/t_G) for the SB-90 and

Table 3
Gradient retention times for neutral and ionic solutes and the SB-100 column

Solute	Retention time t_R (min)			
	$t_G = 10$ min 35 °C	$t_G = 20$ min 35 °C	$t_G = 10$ min 50 °C	$t_G = 20$ min 50 °C
1. Benzene	8.213	12.08	7.820	11.28
2. Toluene	9.324	14.40	8.963	13.68
3. Ethylbenzene	10.23	16.21	9.893	15.55
4. *p*-Xylene	10.29	16.32	9.936	15.63
5. Propylbenzene	11.12	17.99	10.76	17.32
6. Butylbenzene	11.92	19.58	11.52	18.85
7. Naphthalene	10.03	15.90	9.652	15.17
8. 4-Chlorotoluene	10.27	16.34	9.920	15.65
9. *p*-Dichlorobenzene	10.09	15.98	9.756	15.28
11. Bromobenzene	9.591	14.95	9.232	14.24
12. 1-Nitropropane	5.429	6.526	5.030	5.950
13. Nitrobenzene	7.638	11.10	7.186	10.23
14. 4-Nitrotoluene	8.657	13.26	8.281	12.47
15. 4-Nitrobenzyl chloride	8.724	13.53	8.298	12.65
16. *N*-benzylformamide	4.827	6.282	4.552	5.788
17. Anisole	8.102	12.01	7.713	11.24
18. Benzyl alcohol	4.940	6.321	4.708	5.900
19. 3-Phenyl propanol	6.698	9.714	6.487	9.274
20. 5-Phenyl pentanol	8.379	12.99	8.149	12.55
21. Phenol	5.316	6.820	4.892	6.078
22. *p*-Chlorophenol	7.049	10.31	6.630	9.484
23. 2,3-Dihydroxynapthalene	6.516	9.508	6.161	8.790
24. 1,3-Dihydroxynapthalene	6.311	9.181	5.913	8.393
25. Eugenol	8.008	12.18	7.735	11.64
26. Danthron	10.16	16.16	9.753	15.37
27. *n*-Propylformate	5.580	6.990	5.272	6.513
28. Methylbenzoate	7.959	11.86	7.611	11.18
29. Benzonitrile	6.949	9.776	6.519	8.957
30. Coumarin	6.393	8.950	6.031	8.289
31. Acetophenone	6.863	9.720	6.526	9.100
32. Benzophenone	9.546	15.08	9.207	14.42
33. *cis*-Chalcone	10.00	16.08	9.668	15.43
34. *trans*-Chalcone	10.29	16.65	9.939	15.97
35. *cis*-4-Nitrochalcone	9.77	15.76	9.424	15.05
36. *trans*-4-Nitrochalcone	10.16	16.54	9.775	15.76
37. *cis*-4-Methoxychalcone	9.855	15.81	9.499	15.18
38. *trans*-4-Methoxychalcone	10.18	16.46	9.804	15.79
39. Prednisone	6.403	9.645	6.258	9.346
40. Hydrocortisone	6.385	9.629	6.293	9.416
41. Mephenytoin	6.521	9.516	6.275	9.050
42. Oxazepam	7.097	10.83	6.973	10.55
43. Flunitrazepam	7.909	12.22	7.703	11.80
44. 5,5-Diphenylhydantoin	6.869	10.40	6.615	9.880
45. *N,N*-dimethylacetamide	2.714	2.832	2.589	2.675
46. Amitryptiline	7.066	11.05	6.939	10.76
47. Diphenhydramine	6.230	9.443	6.031	9.094
48. Propranolol	5.830	8.735	5.695	8.408
49. Nortiptyline	6.932	10.85	6.792	10.53

Table 3. Continued

Solute	Retention time t_R (min)			
	$t_G = 10$ min 35 °C	$t_G = 20$ min 35 °C	$t_G = 10$ min 50 °C	$t_G = 20$ min 50 °C
51. 4-*n*-Pentylaniline	7.624	11.19	8.035	11.70
52. 4-*n*-Hexylaniline	8.965	13.40	9.370	14.05
53. 4-*n*-Heptylaniline	10.35	15.71	10.57	16.33
54. *N*-ethylaniline	3.158	3.510	3.201	3.518
55. 2-Phenyl pyridine	5.417	6.735	5.758	7.172
56. Diclofenac acid	9.527	15.33	9.174	14.69
57. Mefenamic acid	10.27	16.72	9.92	16.08
58. Ketoprofen	8.137	12.75	7.856	12.21
59. Diflunisal	10.27	16.72	9.920	16.08
60. 4-*n*-Butylbenzoic acid	9.256	14.72	8.964	14.16
61. 4-*n*-Pentylbenzoic acid	10.05	16.22	9.715	15.62
63. 3-Cyanobenzoic acid	5.367	7.246	4.949	6.478
64. 2-Nitrobenzoic acid	4.491	5.470	4.048	4.799
65. 3-Nitrobenzoic acid	6.007	8.403	5.537	7.515
66. 2,6-Dimethylbenzoic acid	6.467	9.255	6.166	8.695
67. 2-Fluorobenzoic acid	5.422	7.298	5.050	6.609

For other conditions, see Section 3. Data are uncorrected (see Appendix in Ref. [1]).

Symmetry columns vs. the SB-100 column, based on the gradient data of Table 3–5. These results for the effect of temperature on retention as a function of the column are similar to what was observed for a change in %*B* (Figs. 1 and 2), with smaller SE values in (a) compared to (b). All three columns exhibit very similar values of $B''/t_G = \delta \log k(T)$ for a given solute, suggesting that relative column selectivity does not change when the separation temperature is changed.

4.3. Effect on retention of a change in solvent as a function of the column

Table 6 summarizes data for the gradient separation of selected solutes with three different B solvents (ACN, MeOH and THF) and three different columns (SB-100, SB-90 and Symmetry). Solvent-induced changes in selectivity were calculated, using ACN as the reference solvent:

$$\delta t_R(\text{MeOH}) = t_R(\text{MeOH}) - t_R(\text{ACN}) \quad (8)$$

$$\delta t_R(\text{THF}) = t_R(\text{THF}) - t_R(\text{ACN}) \quad (9)$$

For a given column, $t_R(X)$ refers to the retention time using solvent *X*, while $\delta t_R(X)$ is defined by Eqs. (8) and (9). Fig. 4 shows the correlation of values of δt_R(MeOH) (a,b) and δt_R(THF) (c,d) for either the SB-90 or Symmetry columns vs. corresponding values for the SB-100 column. In each case, a good correlation is observed ($0.976 > r > 0.993$), with slopes close to unity (0.96–1.03). Similarly, the standard error of these plots is small (SE = 0.12–0.18).

4.4. Summary of changes in column selectivity with a change in %B, temperature or solvent

The correlations of Figs. 1, 3 and 4 are summarized in Table 7. The standard error (SE) of these correlations ($y = ax$, zero-intercept forced) are listed in the fourth column of data, while the standard deviation (SD) in *y* as a result of these changes in conditions is shown in the fifth column. The ratio SE/SD (last column in Table 7) thus represents the relative importance of (a) change in selectivity due to the effect of conditions on column selectivity as measured by SE vs. (b) change in selectivity due to a change of the conditions per se (e.g. $\delta \log k$[T] values for a given column) as measured by SD. It is

Table 4
Gradient retention times for neutral and ionic solutes and the SB-90 column

Solute	Retention time t_R (min)			
	$t_G = 10$ min 35 °C	$t_G = 20$ min 35 °C	$t_G = 10$ min 50 °C	$t_G = 20$ min 50 °C
1. Benzene	8.218	12.05	7.824	11.27
2. Toluene	9.331	14.34	8.957	13.63
3. Ethylbenzene	10.17	16.13	9.855	15.46
4. *p*-Xylene	10.24	16.22	9.893	15.55
5. Propylbenzene	11.03	17.85	10.69	17.21
6. Butylbenzene	11.82	19.40	11.44	18.71
7. Naphthalene	10.02	15.87	9.658	15.15
8. 4-Chlorotoluene	10.27	16.26	9.918	15.55
9. *p*-Dichlorobenzene	10.09	15.95	9.732	15.25
11. Bromobenzene	9.564	14.92	9.209	14.17
12. 1-Nitropropane	5.476	6.501	5.082	6.013
13. Nitrobenzene	7.710	11.19	7.272	10.35
14. 4-Nitrotoluene	8.714	13.33	8.326	12.56
15. 4-Nitrobenzyl chloride	8.779	13.60	8.355	12.75
16. *N*-benzylformamide	4.968	6.481	4.671	6.016
17. Anisole	8.157	12.03	7.756	11.28
18. Benzyl alcohol	5.036	6.432	4.788	6.018
19. 3-Phenylpropanol	6.824	9.899	6.576	9.426
20. 5-Phenylpentanol	8.513	13.15	8.258	12.71
21. Phenol	5.384	6.888	4.962	6.180
22. *p*-Chlorophenol	7.131	10.39	6.696	9.602
23. 2,3-Dihydroxynapthalene	6.603	9.619	6.227	8.893
24. 1,3-Dihydroxynapthalene	6.381	9.304	5.980	8.516
25. Eugenol	8.039	12.25	7.770	11.71
26. Danthron	10.20	16.21	9.788	15.44
27. *n*-Propylformate	5.590	6.956	5.314	6.536
28. Methylbenzoate	8.029	11.93	7.669	11.30
29. Benzonitrile	7.034	9.892	6.607	9.108
30. Coumarin	6.539	9.130	6.159	8.485
31. Acetophenone	6.950	9.855	6.626	9.213
32. Benzophenone	9.583	15.13	9.249	14.50
33. *cis*-Chalcone	10.02	16.08	9.689	15.49
34. *trans*-Chalcone	10.33	16.67	9.970	16.05
35. *cis*-4-Nitrochalcone	9.818	15.82	9.496	15.15
36. *trans*-4-Nitrochalcone	10.21	16.59	9.849	15.86
37. *cis*-4-Methoxychalcone	9.864	15.86	9.568	15.27
38. *trans*-4-Methoxychalcone	10.20	16.53	9.881	15.90
39. Prednisone	6.538	9.889	6.387	9.588
40. Hydrocortisone	6.523	9.892	6.423	9.642
41. Mephenytoin	6.599	9.649	6.363	9.179
42. Oxazepam	7.194	10.97	7.035	10.66
43. Flunitrazepam	8.031	12.41	7.802	11.98
44. 5,5-Diphenylhydantoin	6.969	10.54	6.690	10.00
45. *N*,*N*-dimethylacetamide	2.805	2.960	2.706	2.815
46. Amitryptiline	7.133	11.18	6.994	10.86
47. Diphenhydramine	6.338	9.549	6.159	9.206
48. Propranolol	5.927	8.86	5.766	8.523
49. Nortiptyline	7.010	10.94	6.856	10.66

Table 4. Continued

Solute	Retention time t_R (min)			
	$t_G = 10$ min 35 °C	$t_G = 20$ min 35 °C	$t_G = 10$ min 50 °C	$t_G = 20$ min 50 °C
51. 4-*n*-Pentylaniline	7.708	11.32	8.116	11.80
52. 4-*n*-Hexylaniline	9.036	13.51	9.424	14.17
53. 4-*n*-Heptylaniline	10.40	15.80	10.61	16.38
54. *N*-ethylaniline	3.223	3.556	3.258	3.587
55. 2-Phenylpyridine	5.533	6.871	5.853	7.309
56. Diclofenac acid	9.529	15.35	9.197	14.75
57. Mefenamic acid	10.27	16.72	9.947	16.07
58. Ketoprofen	8.207	12.83	7.914	12.33
59. Diflunisal	8.806	13.91	8.304	13.06
60. 4-*n*-Butylbenzoic acid	9.297	14.77	8.994	14.22
61. 4-*n*-Pentylbenzoic acid	10.08	16.23	9.749	15.66
63. 3-Cyanobenzoic acid	5.432	7.329	5.011	6.583
64. 2-Nitrobenzoic acid	4.451	5.365	4.053	4.768
65. 3-Nitrobenzoic acid	6.051	8.450	5.590	7.606
66. 2,6-Dimethylbenzoic acid	6.511	9.327	6.217	8.747
67. 2-Fluorobenzoic acid	5.468	7.355	5.108	6.700

For other conditions, see Section 3.

seen that the relative contribution of conditions to column selectivity (a) is small (3–26%); this contribution is smaller for the more similar SB-90 and SB-100 columns (3–14%), and larger for the less similar Symmetry and SB-100 columns (9–26%). This is not unexpected, as in the limiting case of two identical columns the changes in retention with all conditions should be the same for each column. The relative importance of conditions in affecting column selectivity increases in the order %*B* (least)> temperature>solvent type (most).

We conclude that a change in these separation conditions (%*B*, *T*, solvent type) will have little effect on measured values of the column parameters for similar columns (e.g. different batches of nominally equivalent columns), but a somewhat larger effect for columns that are more different. The columns studied here include only C_{18} phases and type-B silica as support; when columns that are more different (type-A silica; C_8, cyano, phenyl, embedded polar group, etc.) are compared, larger changes in column parameters with a change in conditions can be expected. It is important to keep in mind that values of the column parameters ***H***, ***S***, etc. are *relative* to a hypothetical ''average'' column, so a change in conditions will lead to greater changes in ***H***, ***S***, etc. for columns that are more different from each other (or a greater change in their relative selectivities). However, for columns of a *given* type (e.g. C_{18}, C_8, cyano, phenyl, or columns with an embedded polar group) changes in column selectivity with conditions should be similar to the results of Table 7, i.e. little change in column selectivity and values of ***H***, ***S***, etc.

4.5. Effect on retention of a change in pH or buffer concentration (SB-100 column)

Table 8 summarizes changes in $\log k$ ($\delta \log k$) for the SB-100 column as a result of a change in either pH (from 2.7 to 2.9) or buffer concentration (from 31 to 15.5 m*M*). Unreported studies for 50% acetonitrile–buffer as mobile phase suggest that the strong bases (≠46–50) are roughly half-ionized at pH 7, meaning that at pH 2.8 (our standard conditions) the fraction of non-protonated molecules should be negligible ($\approx 10^{-5}$). As a consequence, values of $\log k$ are little affected by a 0.2-unit increase in pH (+0.006–0.009). Presumably, this increase in k at higher pH is due to a slightly increased ionization of column silanols, with increased attraction of the positively charged solute by the negatively charged column. $\log k$ for the strong bases increases

Table 5
Gradient retention times for neutral and ionic solutes and the Symmetry column: temperature and gradient time varied

Solute	Retention time t_R (min)			
	$t_G = 10$ min 35 °C	$t_G = 20$ min 35 °C	$t_G = 10$ min 50 °C	$t_G = 20$ min 50 °C
1. Benzene	8.655	12.88	8.264	12.06
2. Toluene	9.794	15.19	9.421	14.45
3. Ethylbenzene	10.68	17.01	10.26	16.26
4. *p*-Xylene	10.79	17.21	10.39	16.42
5. Propylbenzene	11.59	18.84	11.15	18.05
6. Butylbenzene	n.d.	n.d.	n.d.	n.d.
7. Naphthalene	10.44	16.63	10.02	15.85
8. 4-Chlorotoluene	10.75	17.13	10.34	16.42
9. *p*-Dichlorobenzene	10.58	16.83	10.18	16.00
11. Bromobenzene	10.05	15.78	9.632	14.98
12. 1-Nitropropane	5.742	7.028	5.354	6.402
13. Nitrobenzene	7.846	11.50	7.396	10.64
14. 4-Nitrotoluene	8.892	13.68	8.456	12.81
15. 4-Nitrobenzylchloride	8.906	13.86	8.49	12.97
16. *N*-benzyl-formamide	9.017	13.38	9.44	14.09
17. Anisole	8.445	12.58	8.058	11.82
18. Benzyl alcohol	5.129	6.638	4.855	6.134
19. 3-Phenyl propanol	6.875	10.02	6.648	9.52
20. 5-Phenyl pentanol	8.533	13.22	8.316	12.80
21. Phenol	5.588	7.297	5.177	6.523
22. *p*-Chlorophenol	7.339	10.83	6.948	10.03
23. 2,3-Dihydroxynapthalene	6.745	9.924	6.348	9.148
24. 1,3-Dihydroxynapthalene	6.511	9.553	6.094	8.737
25. Eugenol	8.245	12.61	7.934	12.03
26. Danthron	10.51	16.77	9.965	15.76
27. *n*-Propyl formate	n.d.	n.d.	n.d.	n.d.
28. Methylbenzoate	8.18	12.21	7.839	11.54
29. Benzonitrile	7.116	10.06	6.699	9.239
30. Coumarin	6.423	8.94	6.081	8.313
31. Acetophenone	7.008	9.941	6.665	9.314
32. Benzophenone	9.713	15.37	9.363	14.67
33. *cis*-Chalcone	10.14	16.31	9.812	15.65
34. *trans*-Chalcone	10.44	16.89	10.08	16.19
35. *cis*-4-Nitrochalcone	n.d.	n.d.	n.d.	n.d.
36. *trans*-4-Nitrochalcone	n.d.	n.d.	n.d.	n.d.
37. *cis*-4-Methoxychalcone	n.d.	n.d.	n.d.	n.d.
38. *trans*-4-Methoxychalcone	n.d.	n.d.	n.d.	n.d.
39. Prednisone	6.279	9.384	6.094	9.092
40. Hydrocortisone	6.28	9.446	6.212	9.239
41. Mephenytoin	n.d.	n.d.	n.d.	n.d.
42. Oxazepam	7.253	11.10	7.104	10.74
43. Flunitrazepam	7.959	12.32	7.705	11.83
44. 5,5-Diphenylhydantoin	7.009	10.65	6.702	10.05
45. *N*,*N*-dimethylacetamide	2.511	2.543	2.38	2.397
46. Amitryptiline	6.279	9.837	6.094	9.466
47. Diphenhydramine	5.605	8.394	5.412	8.025
48. Propranolol	5.318	7.905	5.079	7.441
49. Nortiptyline	6.206	9.668	6.043	9.335

Table 5. Continued

Solute	Retention time t_R (min)			
	t_G = 10 min 35 °C	t_G = 20 min 35 °C	t_G = 10 min 50 °C	t_G = 20 min 50 °C
51. 4-*n*-Pentylaniline	7.515	10.95	7.945	11.52
52. 4-*n*-Hexylaniline	n.d.	n.d.	n.d.	n.d.
53. 4-*n*-Heptylaniline	10.54	15.85	10.71	16.49
54. *N*-ethylaniline	2.931	3.0915	2.949	3.113
55. 2-Phenyl pyridine	5.347	6.57	5.692	6.966
56. Diclofenac acid	n.d.	n.d.	n.d.	n.d.
57. Mefenamic acid	10.51	17.13	10.13	16.42
58. Ketoprofen	8.265	12.95	7.994	12.41
59. Diflunisal	9.424	14.78	8.88	13.81
60. 4-*n*-Butylbenzoic acid	9.543	15.21	9.199	14.62
61. 4-*n*-Pentylbenzoic acid	10.39	16.76	10.03	
63. 3-Cyanobenzoic acid	5.524	7.487	5.079	6.668
64. 2-Nitrobenzoic acid	4.686	5.736	4.282	5.089
65. 3-Nitrobenzoic acid	6.192	8.694	5.747	7.833
66. 2,6-Dimethylbenzoic acid	6.713	9.673	6.359	9.074
67. 2-Fluorobenzoic acid	5.588	7.584	5.177	6.904

For other conditions, see Section 3.

(+0.039–0.049) for a 2-fold reduction in buffer concentration, presumably due to the decreased competition of buffer cations for interaction (ion-exchange) with ionized silanols ($-SiO^-$) in the stationary phase.

For a change in pH from 2.7 to 2.9, larger (more variable) changes in log *k* are found for the weak acids (≠56–67) and bases (≠51–55), relative to the behavior of the strong bases. The effect of an increase in pH is to increase log *k* for the weak bases (average $\delta \log k$[pH] = 0.13±0.04) and to decrease log *k* for the weak acids (average $\delta \log k$[pH] = 0.019±0.030), in agreement with the expected changes in degree of ionization of these compounds with pH. By means of the Henderson–Hasselbach equation [10], it is possible to estimate the charge on solutes ≠56–67 with a mobile phase pH 2.80 (last column of Table 8). The latter calculation assumes that only the uncharged solute is retained (except for totally ionized solutes ≠46–50), and any change in silanol ionization is ignored.

Average values of log *k* for the weak bases (≠51–55) show a small *decrease* as the buffer is decreased from 31.2 to 15.6 m*M* (Table 8): −0.014±0.005, while weak acids (≠56–67) show a similar but opposite *increase*: +0.009±0.009. These changes in retention are counter-intuitive, since ion-exchange based retention of the cationic bases should increase with decreasing buffer concentration, while enhanced Donnan exclusion of anions is expected to increase the retention of ionized acids at higher buffer concentrations. Fig. 5 compares these changes in retention with decreased buffer concentration ($\delta \log k$[buffer]) vs. changes for increased pH ($\delta \log k$[pH]). The latter changes in *k* with buffer concentration and the correlations of Fig. 5 are consistent with a variation in the pK_a values of these acids and bases with ionic strength; if true, this means that buffer-related changes in *k* should be similar for different columns, with little effect on values of ***H***, ***S***, etc.

*4.5.1. Effect of pH on silanol ionization, solute retention and values of the column parameter **C***

The effect of silanol ionization on the retention of (fully ionized) cationic solutes is described in Eq. (7) of Section 2, i.e.:

$$\log k \approx c_x + \boldsymbol{C}$$

For a given solute *x*, c_x is a constant, and ***C*** will be a function of the concentration of *accessible* ionized silanols; ***C*** is expected to increase as pH and silanol ionization increase. A simple interpretation of Eq. (7) is that different univalent solute cations exhibit

Table 6
Gradient retention times for change of solvent (neutral and ionic solutes)

Solute	Change in retention time δt_R (min)					
	MEOH replaces ACN[a]			THF replaces ACN[a]		
	SB-100	SB-90	Symmetry	SB-100	SB-90	Symmetry
7. Naphthalene	1.527	1.481	1.627	−1.733	−1.691	−2.037
10. Benzotrichloride	2.106	2.006	2.41	0.407	0.392	0.65
13. Nitrobenzene	0.786	0.802	0.865	−0.624	−0.622	−0.705
14. 4-Nitrotoluene	1.1	1.117	1.17	−1.052	−1.036	−1.13
16. *N*-benzylformamide	1.541	1.498	1.429	−0.958	−1.011	−0.917
18. Benzyl alcohol				−0.42	−0.402	−0.373
20. 5-Phenyl pentanol				−1.266	−1.32	−1.223
21. Phenol	0.959	0.907	1.038			
22. *p*-Chlorophenol	1.706	1.619	1.882	0.281	0.264	0.179
23. 2,3-Dihydroxynaphthalene	1.717	1.665	1.796	0.43	0.407	0.343
24. 1,3-Dihydroxynaphthalene	1.674	1.612	1.791	0.486	0.456	0.474
25. Eugenol	1.827	1.813	1.81			
26. Danthron				−1.807	−1.8	−2.07
30. Coumarin	1.358	1.306	1.109	−1.182	−1.223	−1.1
32. Benzophenone	1.402	1.382	1.259	−1.735	−1.716	−1.839
33. *cis*-Chalcone	1.238	1.196	1.118	−1.767	−1.918	−1.976
34. *trans*-Chalcone	1.447	1.415	1.357			
39. Prednisone	3.028	3.031	2.742	−0.99	−1.031	−0.713
40. Hydrocortisone	3.428	3.417	3.245	−0.616	−0.652	−0.406
42. Oxazepam	2.932	2.891	2.847	−0.801	−0.795	−0.782
43. Flunitrazepam	1.652	1.679	1.631	−1.245	−1.26	−1.163
44. 5,5-Diphenylhydantoin	2.148	2.115	2.123	−0.174	−0.207	−0.133
45. *N*,*N*-dimethylacetamide	0.881	0.886	0.648			
46. Amitryptiline	2.731	2.663	2.604	−1.512	−1.765	−1.08
48. Propranolol	2.469	2.476	1.928	−0.712	−0.879	−0.722
49. Nortiptyline	3.075	3.02	2.776	−1.148	−1.384	−0.828
52. 4-*n*-Hexylaniline	2.676	2.819	2.451	−1.037	−0.927	−1.177
53. 4-*n*-Heptylaniline				−1.798	−1.648	−2.161
55. 2-Phenyl pyridine	2.914	3.266	2.392	−0.349	0.075	−0.439
57. Mefenamic acid				−1.691	−1.681	−1.814
58. Ketoprofen	2.261	2.203	2.131			
60. 4-*n*-Butylbenzoic acid	2.487	2.467	2.428	−1.121	−1.108	−1.29
61. 4-*n*-Pentylbenzoic acid				−1.016	−1.05	−1.219
63. 3-Cyanobenzoic acid	1.571	1.573	1.738	0.716	0.728	0.786
64. 2-Nitrobenzoic acid	0.291	0.028	0.442	0.206	−0.009	−0.053
65. 3-Nitrobenzoic acid	1.65	1.556	1.804	0.866	0.876	0.658
66. 2,6-Dimethylbenzoic acid	2.069	2.01	2.199	0.205	0.178	0.119
67. 2-Fluorobenzoic acid	1.627	1.55	1.602	0.419	0.385	0.304

Gradient time $t_G = 10$ min, 35 °C. For other conditions, see Section 3.

the same electrostatic interaction with ionized silanols, and this (constant) interaction energy for a given pH and column is then added to the total free energy of retention for the solute. The validity of Eq. (7) can be assessed in the following way. For different univalent cationic solutes and a given column, let values of log k vs. pH be plotted on the same graph—then construct a best-fit curve through data points for one of the solutes. If Eq. (7) is valid (only for solutes whose ionization does not change), this same curve can be fit to the data for other solutes by a simple vertical shift (corresponding to different values of the quantity c_x of Eq. (7) for different solutes).

The above test of Eq. (7) is illustrated by the data of Fig. 6, where values of log k are plotted vs. pH for

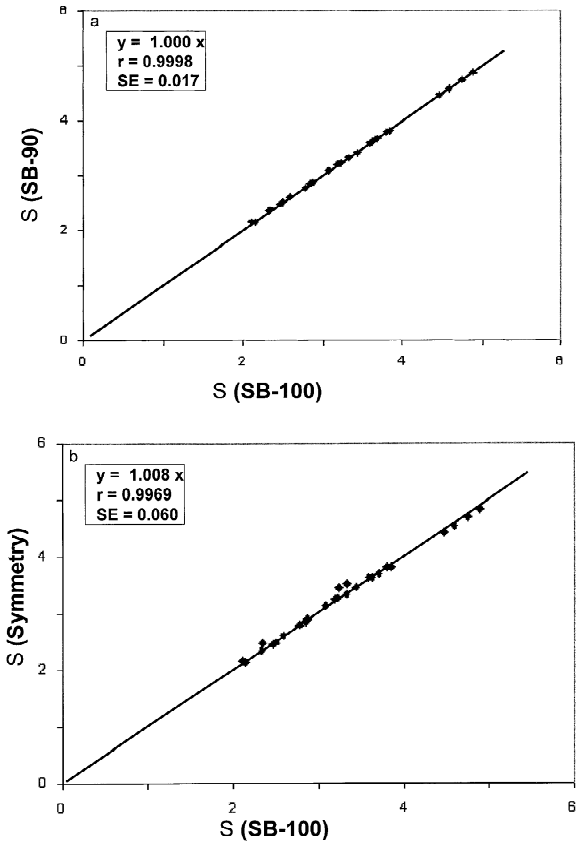

Fig. 1. Effect of a change in % B on column selectivity. Correlation of values of $S = \mathrm{d}(\log k)/\mathrm{d}\phi$ among different columns. Values of S calculated (via Eq. (3)) from isocratic data of Table 2 for 27 neutral solutes; other conditions as in Section 3. (a) S-values for SB-90 column plotted vs. values for SB-100 column; (b) S-values for Symmetry column plotted vs. values for SB-100 column. See text for details.

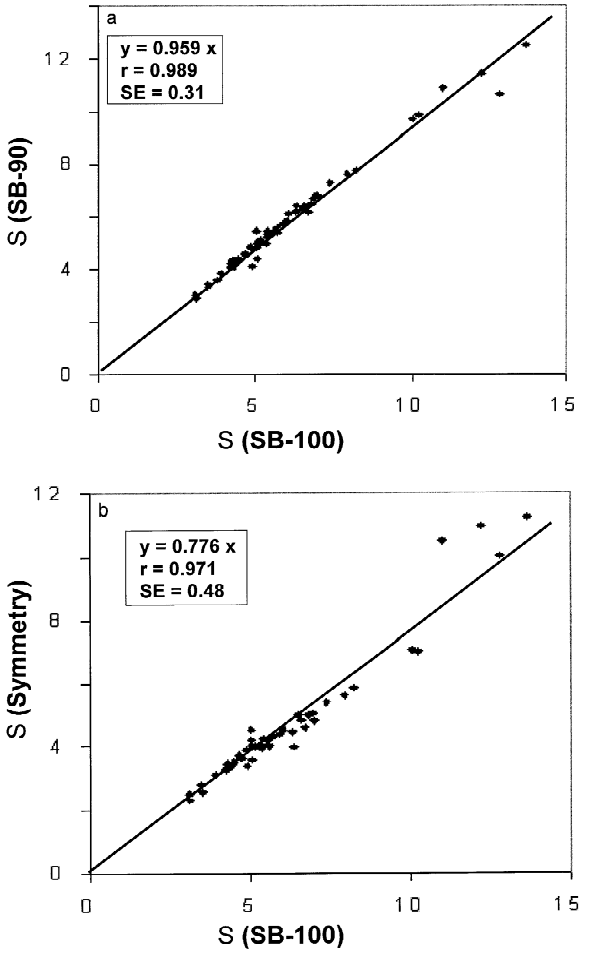

Fig. 2. Effect of a change in gradient time t_G on column selectivity. Correlation of values of $S = \mathrm{d}(\log k)/\mathrm{d}\phi$ among different columns. Values of S calculated from gradient data of Tables 3–5 for 67 neutral, acidic and basic solutes; other conditions as in Section 3. (a) S-values for SB-90 column plotted vs. values for SB-100 column; (b) S-values for Symmetry column plotted vs. values for SB-100 column. See text for details.

two strong bases (≠46, 49) and two quaternary ammonium compounds (≠91,92). In order to provide a continuous change in pH over the range $3 \leq \mathrm{pH} \leq 7$ without a change in the buffer anion, citrate was used as buffer (instead of the phosphate buffer used for all other experiments reported here and in Parts I and III [1,2]) while maintaining counter-ion (Na^+) concentration constant. Data for three different columns are shown in Fig. 6: (a) Symmetry, (b) SB-100 and (c) Inertsil. In Fig. 6a, the solid curve through the filled circles (quaternary ammonium solute berberine) is a subjective best fit to these data. This same curve is then displaced vertically to provide as close a (subjective) fit as possible to the data for the remaining three solutes in the range $3 \leq \mathrm{pH} \leq 5$. In the latter pH range, all four solutes are expected to be fully protonated. If Eq. (7) applies with $\kappa' \approx$ constant for the four different solutes, the fit of data for each solute over the range $3 \leq \mathrm{pH} \leq 5$ should be equally good, and this appears to be the case. However, for $\mathrm{pH} > 5$ (arrows), the retention for amitriptyline (diamonds) is greater than predicted by the dotted curve, presumably corresponding to re-

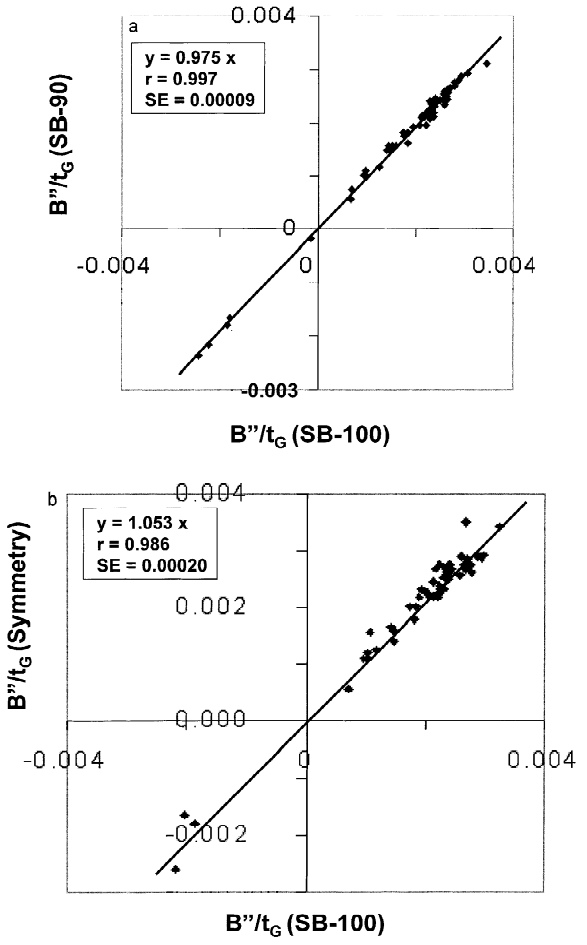

Fig. 3. Effect of a change in temperature on column selectivity. Correlation of values of B''/t_G among different columns. Values of B''/t_G calculated from gradient data of Tables 3–5 for 67 neutral, acidic and basic solutes; other conditions as in Section 3. (a) B''/t_G values for SB-90 column plotted vs. values for SB-100 column; (b) B''/t_G values for Symmetry column plotted vs. values for SB-100 column. See text for details.

duced ionization of the molecule with increasing pH and a resulting increase in retention. A similar, smaller deviation from the predicted curve is noted for nortriptyline at pH 7, suggesting that nortriptyline is fully ionized for $3 \leq pH \leq 6$, but begins to deprotonate significantly for $pH > 6$.

In Fig. 6a for the Symmetry column, there is a small increase in log k for berberine as pH increases from 3 to 7, suggesting that the concentration of *accessible* ionized silanols changes only slightly in this pH range. Similar results for a Symmetry column (from a different production batch) have been reported elsewhere (see Fig. 2b of Ref. [13]). For the SB-100 column (Fig. 6b), values of log k for berberine begin to increase significantly for $pH > 5$. The berberine data suggest an increasing concentration of accessible ionized silanols at higher pH, as expected for a silanol pK_a value of about 7. The Inertsil column (Fig. 6c) shows a modest *decrease* in log k for berberine as pH increases, which is puzzling, i.e. the concentration of accessible ionized silanols is not expected to decrease with increase in pH. It is possible that changes in pH can affect the stationary phase in such a way as to reduce the access of cationic solutes to ionized silanols. However, it should be noted that when phosphate is used instead of citrate as buffer, a small *increase* in berberine retention on the Inertsil column (≠1) is found for pH 7 vs. pH 2.8 (see the following discussion of Table 9). The greater scatter of data points for bicuculline in Fig. 6c can be attributed to very small values of k, with a greater error in their determination.

4.5.2. Measurement of *C* as a function of pH

From the above discussion, it can be seen that Eq. (7) provides a means for determining values of ***C*** as a function of pH. Given a value of ***C*** at pH 2.8 (***C***[pH 2.8]), values of k for the quaternary ammonium solute berberine can be determined as a function of pH. For any $pH = x$, ***C*** for pH x (***C***[pH x]) is given as:

$$\boldsymbol{C}(\text{pH } x) = \boldsymbol{C}(\text{pH } 2.8) + \log(k_x/k_{2.8}) \quad (10)$$

where k_x and $k_{2.8}$ refer to values of k for $pH = x$ and 2.8, respectively. It is seen in Fig. 6 that the assumption of a linear dependence of ***C*** on pH in the range $2.8 < pH < 7$ is an acceptable first approximation for the purpose of estimating values of ***C*** at intermediate pH values. We propose to measure values of ***C*** for pH 2.8 and 7.0 (Eq. (10)) and assume a linear interpolation for other pH values.

The change in ***C*** as mobile phase pH is increased from 2.8 to 7.0 is shown for several columns in Table 9. In all cases but one, the retention of berberine (and values of ***C***) increases at higher pH, as expected; column ≠7 exhibits a small decrease in ***C***, which may in part be due to experimental error.

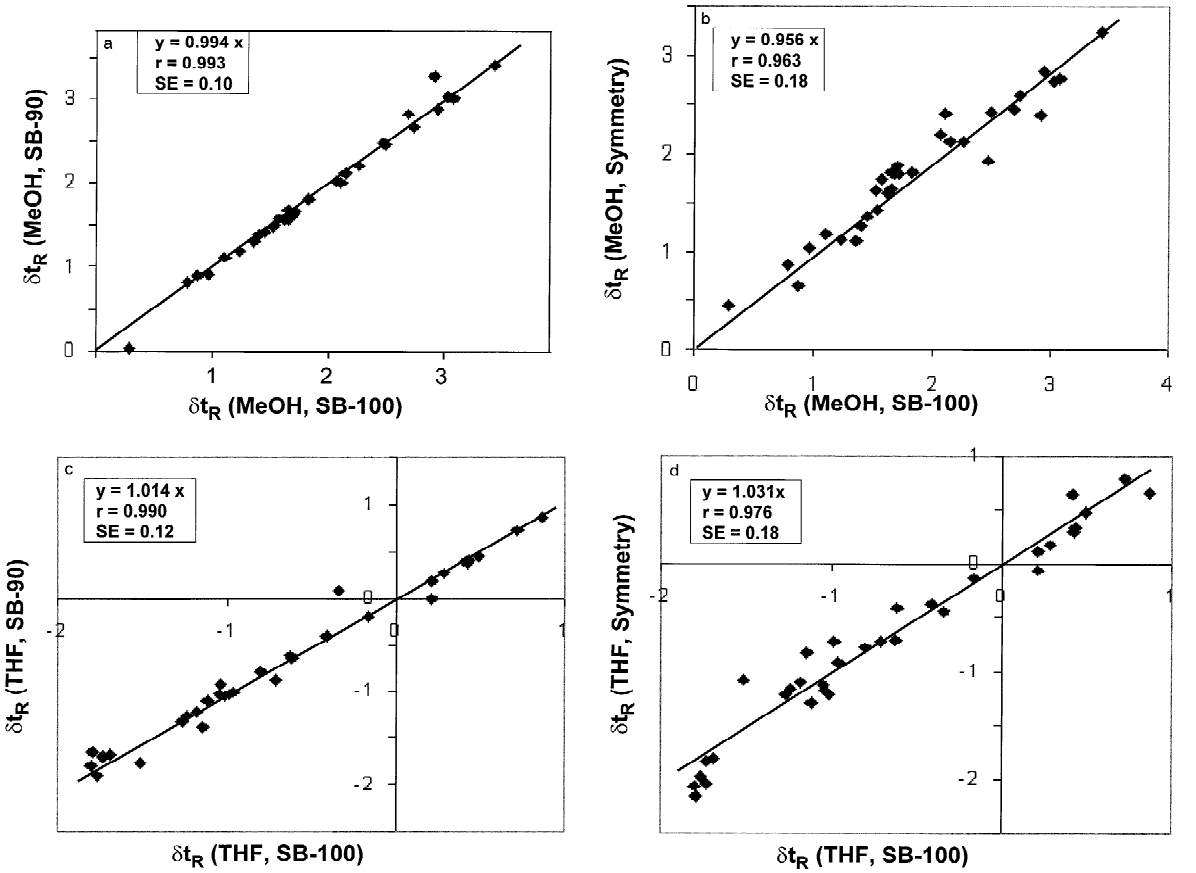

Fig. 4. Effect of a change in solvent on column selectivity; new solvent replaces ACN, correlation of values of δt_R between different columns. Values of δt_R calculated via Eqs. (8) or (9) (data of Table 6); 5–81%*B* gradients in 10 min, other conditions as in Section 3. (a) δt_R values for MeOH vs. ACN, SB-90 vs. SB-100 columns; (b) δt_R values for MeOH vs. ACN, Symmetry vs. SB-100 columns; (c) δt_R values for THF vs. ACN, SB-90 vs. SB-100 columns; (d) δt_R values for THF vs. ACN, Symmetry vs. SB-100 columns. See text for details.

As in the examples of Fig. 6, the relative increase in berberine retention (and values of ***C***) with pH varies widely for the columns of Table 9. Note that the biggest changes in ***C*** occur for the non-end-capped columns #3 and 4 (***C*** increases by 0.73–0.76), whereas the end-capped columns have much smaller changes (−0.05 to 0.16). This behavior is expected, assuming that end-capping eliminates some ionizable silanols and blocks the interaction of cationic solutes with other (unreacted and ionized) silanols.

5. Conclusions

The preceding paper [1] describes a model for column selectivity that allows quantitative predictions of k as a function of the properties of a given solute and column. For a given set of experimental conditions (temperature, mobile phase composition), column selectivity is defined by five parameters: ***H***, ***S***, ***A***, ***B*** and ***C***. If changes in $\log k$ as a result of changes in experimental conditions are the same for a given solute with different columns, then column selectivity and relative values of the parameters ***H***, ***S***, etc. will not change as conditions are varied. Experimental results from this study show similar changes in $\log k$ (or gradient retention time) for all three columns with temperature, solvent strength (either isocratic % *B* or gradient time), or solvent type (acetonitrile, methanol, tetrahydrofuran). Thus, values of the selectivity parameters ***H***, ***S***, etc. obtained for a given column under one set of

Table 7
Summary of effects of changes in conditions on solute retention

Condition, column	Correlation data				
	Slope a ($y = ax$)[a]	r	SE	SD	SE/SD (%)
% B (values of $S = \mathrm{d}\log k/\mathrm{d}\phi$)[b] (Fig. 1)					
SB-90	1.00	1.000	0.02	0.64	3
Symmetry	1.01	0.997	0.06	0.65	9
Temperature (values of B''/t_G) (Fig. 3)					
SB-90	0.98	0.997	0.0001	0.0013	8
Symmetry	1.05	0.986	0.0002	0.0013	15
MeOH replaces ACN (values of δt_R)[c] (Fig. 4)					
SB-90	0.994	0.993	0.10	0.79	13
Symmetry	0.956	0.963	0.18	0.68	26
THF replaces ACN (values of δt_R)[c] (Fig. 4)					
SB-90	1.01	0.990	0.12	0.84	14
Symmetry	1.03	0.976	0.18	0.86	21

Correlations for SB-90 and Symmetry columns vs. SB-100 column (data of Figs. 1, 3, and 4).
SE, standard error of correlation; SD, standard deviation of values for different solutes, as a result of change in the designated variable.
[a] Intercept forced to zero.
[b] Isocratic data (Table 2, Fig. 1).
[c] From Eqs. (9) or (10).

conditions (% B, temperature, etc.) can be used to characterize that column's selectivity for other conditions.

The preceding conclusion considerably simplifies the task of characterizing column selectivity, as measurements of solute retention are needed only for a single temperature and mobile phase composition (except for pH). As expected, column-to-column differences for a change in $\log k$ with conditions become smaller, the more similar two columns are. The latter observation provides a conditions-independent basis for the quality control of RP-LC column selectivity by the manufacturer. Thus, different production batches of a stationary phase (which should be similar, but not necessarily identical) which have ''sufficiently'' similar values of $\boldsymbol{H}$, $\boldsymbol{S}$, etc. should provide equivalent retention and separation for other samples and conditions. It may be unnecessary to calculate actual values of $\boldsymbol{H}$, $\boldsymbol{S}$, etc. for the testing of different column batches. As with present column-manufacturing practice, it may suffice to simply measure retention (or retention ratios vs. toluene or ethylbenzene) for solutes which have large values of each column parameter, e.g. amitriptyline as an indicator of the parameter $\boldsymbol{C}$.

Preliminary measurements were carried out for the retention of ionizable solutes as a function of mobile phase pH and buffer concentration (for selected neutral molecules, a change in pH from 3 to 7 resulted in little change in values of k [$<2\%$]). The major contribution of pH and buffer concentration to change in solute retention and values of the column parameters ($\boldsymbol{H}$, $\boldsymbol{S}$, etc.) is likely to be the same for different columns, with the exception of the column parameter $\boldsymbol{C}$ (which measures the ion-exchange retention of protonated bases by ionized silanols [2]). The dependence of $\boldsymbol{C}$ on mobile phase pH can be determined using a quaternary ammonium compound as solute (e.g. berberine), similar to the procedure of Neue et al. [13].

6. Glossary of terms and acknowledgements

See Part I [1].

Appendix A. Effect of a change in conditions on solute or column parameters

A reviewer's analysis was as follows. First, assume that a change in the solute parameters ($\delta\eta'$, $\delta\sigma'$, etc.) takes place as a result of some change in experimental conditions, while the column parameters ($\boldsymbol{H}$, $\boldsymbol{S}$, etc.) remain constant. Let the two columns be designated by subscripts 1 and 2, with initial conditions designated by subscript A, and changed conditions by subscript B. Then, if the change in $\log k$ ($\delta \log k$) with conditions for any solute is the same for columns 1 and 2, we must have

$$\begin{aligned}\delta \log k_1 &= \delta \log k_{\mathrm{ref1}} + \delta\eta'_1 \boldsymbol{H}_1 + \delta\sigma'_1 \boldsymbol{S}_1 + \delta\beta'_1 \boldsymbol{A}_1 \\ &\quad + \delta\alpha'_1 \boldsymbol{B}_1 + \delta\kappa'_1 \boldsymbol{C}_1 \\ &= \delta \log k_2 = \delta \log k_{\mathrm{ref2}} + \delta\eta'_2 \boldsymbol{H}_2 + \delta\sigma'_2 \boldsymbol{S}_2 \\ &\quad + \delta\beta'_2 \boldsymbol{A}_2 + \delta\alpha'_2 \boldsymbol{B}_2 + \delta\kappa'_2 \boldsymbol{C}_2 \qquad \text{(A.1)}\end{aligned}$$

But the latter relationship requires $\delta \log k_{\mathrm{ref1}} = \delta \log k_{\mathrm{ref2}}$, $\delta\eta'_1 = \delta\eta'_2$ (and similarly for $\delta\sigma$, $\delta\beta'$, $\delta\alpha'$ and $\delta\kappa'$), and $\boldsymbol{H}_1 = \boldsymbol{H}_2$ (and similarly for $\boldsymbol{S}$, $\boldsymbol{A}$, $\boldsymbol{B}$ and $\boldsymbol{C}$). That is, the two columns must have identical values

Table 8
Change in log k for various ionic solutes as a result of mobile phase pH or buffer concentration (SB-100 column)

Solute	Change in log k (δ log k)		Estimated charge on molecule[a]
	pH 2.7→2.9	31.2→15.6 mM buffer	
46. Amitriptyline	0.007	0.039	+1.00
47. Diphenhydramine	0.007	0.039	+1.00
48. Propranolol	0.009	0.049	+1.00
49. Nortiptyline	0.006	0.042	+1.00
50. Prolintane	0.012	0.038	+1.00
51. 4-n-C_5 Aniline	0.131	−0.012	+0.71
52. 4-n-C_6 Aniline	0.129	−0.011	+0.71
53. 4-n-C_7 Aniline	0.127	−0.012	+0.71
54. N-ethylaniline	0.184	−0.023	+0.90
55. 2-Phenyl pyridine	0.069	−0.012	+0.61
56. Diclofenac acid	−0.003	0.005	−0.02
57. Mefenamic acid	−0.003	0.005	−0.02
58. Ketoprofen	−0.002	0.004	−0.01
59. Diflunisal	−0.062	0.025	−0.37
60. 4-n-C_4 Benzoic acid	−0.002	0.003	−0.01
61. 4-n-C_5 Benzoic acid	−0.001	0.006	0.00
62. 4-n-C_6 Benzoic acid	−0.001	0.008	0.00
63. 3-Cyanobenzoic acid	−0.015	0.006	−0.09
64. 2-Nitrobenzoic acid	−0.098	0.032	−0.55
65. 3-Nitrobenzoic acid	−0.016	0.007	−0.10
66. 2,6-Dimethylbenzoic acid	−0.012	0.003	−0.08
67. 2-Fluorobenzoic acid	−0.013	0.005	−0.08

For original (''standard'') conditions and other details, see Section 3.
[a] Estimated from Henderson–Hasselbach equation, assuming only neutral molecule is retained (except for strong bases ≠46–50).

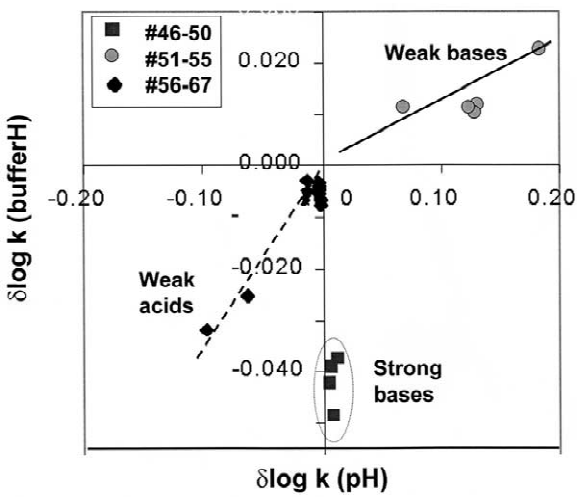

Fig. 5. Change in log k due to a decrease in buffer concentration (δ log k[buffer]) vs. changes in log k due to an increase in pH for the acids and bases of Table 8.

of $\boldsymbol{H}$, $\boldsymbol{S}$, etc. Thus, for Eq. (A.1) to hold, the two columns must be identical in terms of selectivity, a conclusion which is not of any practical help.

A less restrictive result is obtained if we assume that a change in conditions leads to changes in the column parameters (H, S, etc.). In this case, for equal values of δ log k, we require that

$$\begin{aligned}\delta \log k_1 &= \delta \log k_{\mathrm{ref1}} + \eta'_1 \delta \boldsymbol{H}_1 + \sigma'_1 \delta \boldsymbol{S}_1 \\ &\quad + \beta'_1 \delta \boldsymbol{A}_1 + \alpha'_1 \delta \boldsymbol{B}_1 + \kappa'_1 \delta \boldsymbol{C}_1 \\ &= \delta \log k_2 = \delta \log k_{\mathrm{ref2}} + \eta'_2 \delta \boldsymbol{H}_2 + \sigma'_2 \delta \boldsymbol{S}_2 \\ &\quad + \beta'_2 \delta \boldsymbol{A}_2 + \alpha'_2 \delta \boldsymbol{B}_2 + \kappa'_2 \delta \boldsymbol{C}_2 \end{aligned} \quad \text{(A.2)}$$

For Eq. (A.2) to hold, we require δ log $k_{\mathrm{ref1}} = \delta$ log k_{ref2}, $\eta'_1 = \eta'_2$ (and similarly for $\delta\sigma$, $\delta\beta'$, $\delta\alpha'$ and $\delta\kappa'$), and $\delta \boldsymbol{H}_1 = \delta \boldsymbol{H}_2$ (and similarly for $\delta \boldsymbol{S}$, $\delta \boldsymbol{A}$, $\delta \boldsymbol{B}$ and $\delta \boldsymbol{C}$). That is, we require the same values of the solute parameters for the two columns, and the same *changes* in column parameters as a result of a change

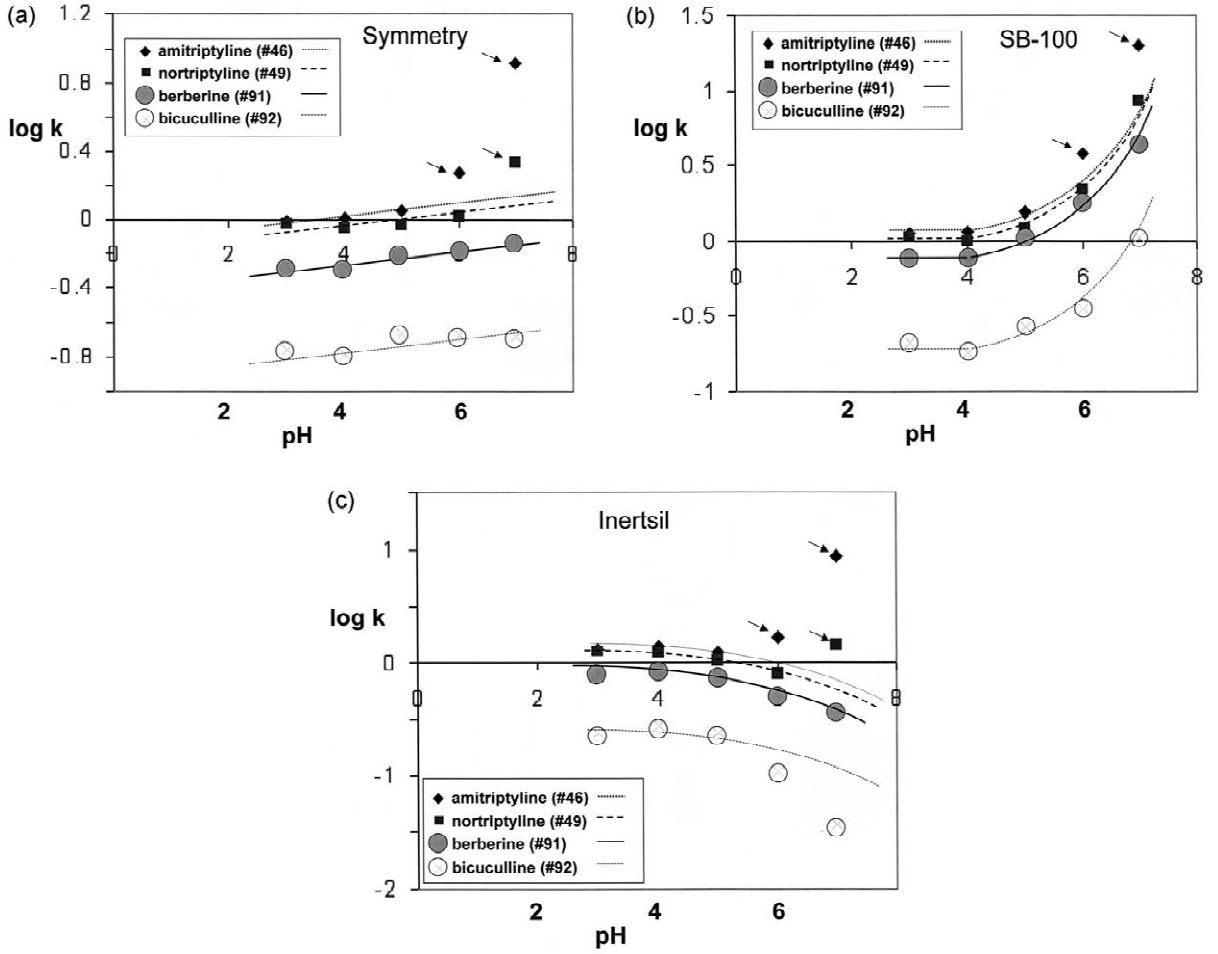

Fig. 6. Retention as a function of pH for cationic solutes. Conditions: 50% acetonitrile–buffer, buffer is 60 m*M* sodium citrate adjusted to given pH; 35 °C; 1.5 ml/min. (a) Symmetry column, (b) SB-100 column, (c) Inertsil column. Solutes indicated in each figure. The subjective, best-fit curve through the berberine data (solid curve) is shifted vertically to overlay data for the other solutes. See text for details.

Table 9
Values of the column parameter ***C*** as a function of pH

Column	***C***		$C_{7.0} - C_{2.8}$ [a]
	pH 3	pH 7	
1. Inertsil	−0.35	−0.32	0.03
2. Symmetry	−0.21	−0.05	0.16
3. SB-100	0.09	0.85	0.76
4. SB-90	0.05	0.78	0.73
6. Eclipse	0.04	0.17	0.13
7. YMC 15	−0.10	−0.15	−0.05
8. YMC 16	0.01	0.02	0.01

[a] Equal to log *k*(pH 7.00) − log *k*(pH 2.80) for berberine (≠91) as solute, determined for 40% ACN–buffer (30 m*M* phosphate buffer); see Eq. (10).

in conditions. But we do not require that the columns initially be the same in terms of selectivity.

The above analysis needs to be considered in light of the fact that values of ***H***, ***S***, etc. are *relative*, not absolute. Thus, differences in column selectivity are defined by differences in their values of ***H***, ***S***, etc. Consequently, if a chnage in conditions results in the same change in each column parameter for different columns, no change in *relative* column selectivity has resulted. Therefore, a determination of values of ***H***, ***S***, etc. at one set of conditions defines relative selectivity for other conditions as well.

Appendix B. Differences in values of $S = d(\log k)/d\phi$ measured by isocratic vs. gradient elution

We have noted in our comparison of Figs. 1 and 2 that values of S measured isocratically (Fig. 1) are the same for a given solute and different columns, whereas plots of S from gradient experiments (Fig. 2) for one column vs. another give slopes that may differ from unity. The reason for this difference in behavior of isocratic vs. gradient data can be explained as follows. First, when using acetonitrile–buffer mobile phases, Eq. (3) provides at best only an approximate description of retention as a function of $\%B$ [3,14,15]. When acetonitrile is used as the B-solvent, plots of $\log k$ vs. ϕ are usually significantly curved, with values of S (measured as the tangent to these plots) decreasing for larger ϕ and smaller k.

In gradient elution, individual solutes elute at different values of ϕ, which suggests that the ratio of (S-gradient)/(S-isocratic) will decrease with increasing solute retention (larger values of k in isocratic elution). In gradient elution, an average retention factor k^* can be defined [12], comparable to k for isocratic separation:

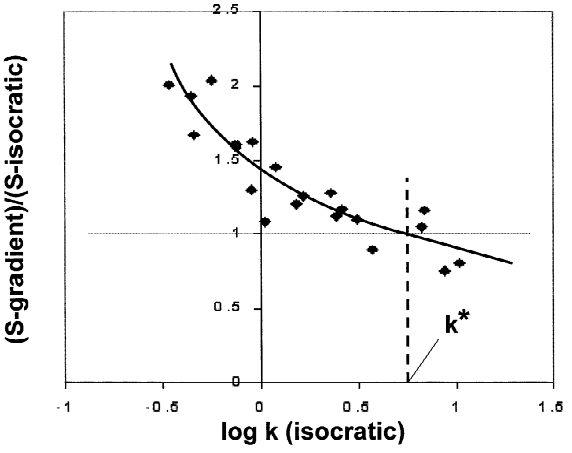

Fig. 7. Ratio of S-values for gradient vs. isocratic separation plotted vs. isocratic retention. See text for details.

$$k^* = (2/2.3)\, t_G F/(V_m \Delta\phi S) \quad \text{(B.1)}$$

Here, F is flow-rate, V_m is the column dead volume, and $\Delta\phi$ is the change in ϕ during the gradient. When k^* (gradient) = k(isocratic), the ratio of (S-gradient)/(S-isocratic) should equal 1, because the average value of ϕ during gradient elution of the solute (ϕ^*) is then equal to the isocratic ϕ-value. This behavior is confirmed in Fig. 7, where $y = (S\text{-gradient})/(S\text{-isocratic})$ is plotted vs. $\log k$ (isocratic values), and it is seen that $y = 1$ for $k^* = k$ (dashed vertical line). The curve through these data points is a subjective best fit.

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