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Optimizing selectivity during reversed-phase high performance liquid chromatography method development: Prioritizing experimental conditions

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ABSTRACT

Several different conditions can be varied to improve selectivity for reversed-phase chromatography (RPC). A reexamination of literature data suggests that changes in selectivity due to a change of column or mobile phase pH are largely replicated by changes in temperature or mobile phase composition (concentrations of acetonitrile and/or methanol). This suggests a reconsideration of the role of mobile phase pH and the column during method development.

1. Introduction

Of the three variables that contribute to resolution (retention factor k, plate number N, separation factor α), optimizing values of α (selectivity) presents both the greatest challenge and greatest opportunity. Empirical approaches for this purpose are now relatively advanced, based on some minimum number of experiments followed by computer simulation [1]. The simultaneous variation of two or more selectivity-influencing conditions has proved especially powerful [2,3]; e.g., temperature, °C, and/or mobile phase composition (i.e., % organic solvent B (%B), ratio of acetonitrile (ACN) and methanol (MeOH), or pH) [2,3]. The present paper proposes a minor fine-tuning of this procedure. Samples which resist improvements in selectivity are discussed first, then such samples are used to expand our understanding of selectivity. Finally the role of the column and mobile phase pH in method development receive special attention. The present study is limited to the optimization of selectivity and does not address related aspects of method development such as ruggedness or Quality by Design (QbD).

2. Experimental

No new experimental data are presented here. A previous study [4] reported isocratic values of k for 67 solutes of widely varying structure (Table 1) and the following conditions: Symmetry C18 column (Waters), 50%v ACN-buffer (31 mM potassium phosphate, pH 2.8) as mobile phase, and 35 °C. Changes in $\log k$ ($\delta \log k$) for these same 67 solutes and column were also reported (Tables 1 and 8 of [5]) for changes in (a) temperature (45 °C vs. 35 °C), (b) ACN concentration (50%v vs. 40%v), (c) replacement of 5%v ACN by 5%v of MeOH to give 45%v ACN/5%v MeOH as mobile phase, and (d) mobile phase pH (3.0 vs. 2.8). Other data from the literature [6,7] were also used.

3. Results and discussion

3.1. "Regular" retention behavior

For a change in concentration of the organic solvent B in the mobile phase, solute retention in RPC can be approximated by

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

Here ϕ is the volume-fraction of B in the mobile phase (equal to $0.01 \times$ %B), k_w is the value of k for $\phi = 0$, and S is a constant for a given solute and organic solvent. Fig. 1a shows plots of $\log k$ vs. %-ACN for benzene and four n-alkylbenzenes. No changes in relative retention

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Table 1
Solutes used in the present study (numbering from [4]).

Solute	Solute	Solute
1. Benzene	24. 1,3-Dihydroxynaphthalene	47. Diphenhydramine
2. Toluene	25. Eugenol	48. Propranolol
3. Ethylbenzene	26. Danthron	49. Nortriptyline
4. *p*-Xylene	27. *n*-Propyl formate	50. Prolintane
5. Propylbenzene	28. Methylbenzoate	51. 4-*n*-Pentylaniline
6. Butylbenzene	29. Benzonitrile	52. 4-*n*-Hexylaniline
7. Naphthalene	30. Coumarin	53. 4-*n*-Heptylaniline
8. 4-Chlorotoluene	31. Acetophenone	54. N-ethylaniline
9. *p*-Dichlorobenzene	32. Benzophenone	55. 2-Phenyl pyridine
10. Benzotrichloride	33. *cis*-Chalcone	56. Diclofenac acid
11. Bromobenzene	34. *trans*-Chalcone	57. Mefenamic acid
12. 1-Nitropropane	35. *cis*-4-Nitro-chalcone	58. Ketoprofen
13. Nitrobenzene	36. *trans*-4-Nitro-chalcone	59. Diflunisal
14. 4-Nitrotoluene	37. *cis*-4-Methoxy-chalcone	60. 4-*n*-Butylbenzoic acid
15. 4-Nitrobenzyl chloride	38. *trans*-4-Methoxy-chalcone	61. 4-*n*-pentylbenzoic acid
16. N-benzylformamide	39. Prednisone	62. 4-*n*-Hexylbenzoic acid
17. Anisole	40. Hydrocortisone	63. 3-Cyanobenzoic acid
18. Benzyl alcohol	41. Mephenytoin	64. 2-Nitrobenzoic acid
19. 3-Phenyl propanol	42. Oxazepam	65. 3-Nitrobenzoic acid
20. 5-Phenyl pentanol	43. Flunitrazepam	66. 2,6-Dimethylbenzoic acid
21. Phenol	44. 5,5-Diphenyl-hydantoin	67. 2-Fluorobenzoic acid
22. *p*-Chlorophenol	45. N,N-dimethylacetamide	
23. 2,3-Dihydroxynapthalene	46. Amitriptyline	

Compounds #1–45 are neutral, compounds #46–50 are completely ionized strong bases, and compounds #51–67 are partly ionized acids or bases (at pH-2.8).

are observed for these compounds; i.e., near-parallel, non-crossing plots with slopes S that increase slightly for more retained solutes. Samples that exhibit behavior as in Fig. 1a have been described as "regular" [1].

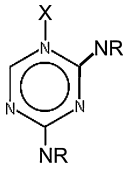
A requirement for "regular" retention behavior as in Fig. 1a is that changes in $\log k$ ($\delta \log k$) for a change in %B must correlate with values of $\log k$ measured prior to any change in conditions.

$$\delta \log k = a + b \log k \quad (2)$$

This is illustrated in Fig. 1b for the same solutes of Fig. 1a. For two solutes that are initially unresolved (separation factor, $\alpha \approx 1$), their values of k will be near-identical. If Eq. (2) applies, their values of $\delta \log k$ will also be approximately equal; a change of %B will thus affect values of k equally, leaving $\alpha \approx 1$. Eq. (2) and Fig. 1b also imply that when $\delta \log k$ and $\log k$ are highly correlated, a change in %B will not change relative peak spacing. Thus, a change in %B is ineffective as a means of improving selectivity for "regular" samples.

"Regular" samples have been described [1] as mixtures of "compounds of highly related structure." This is at best an imprecise description, one that requires elaboration. Fig. 2 provides an example of a "non-regular" sample, a mixture of three alkyl benzenes (○), six alkylbenzenes substituted by a single nitro group (■), and an alkylbenzene substituted by two nitro groups (△). Fig. 2a shows a plot of $\delta \log k$ vs. $\log k$ for this sample. Note that each group of compounds (represented by the same symbol) follows the highly correlated behavior shown in Fig. 1b and Eq. (2), where data points fall on separate curves that are each defined by the number of $-NO_2$ groups in the solute molecule. However, when all the compounds are considered as a single sample, the entire sample is *not* well represented by Eq. (2). The practical consequence of "non-regular" retention behavior for this sample is illustrated in Fig. 2b. Unlike the corresponding plots of Fig. 1a, there are several instances of crossing lines and reversals in relative retention (e.g., for 48%, 51%, 54%, 55% and 59% ACN). While a change in %B for the "regular" sample of Fig. 1 is of little value for improving selectivity, this is not the case for the "non-regular" sample of Fig. 2, where changes in %B cause peaks to move relative to each other.

Some related examples are examined in Fig. 3. Fig. 3a shows a plot of $\delta \log k$ vs. $\log k$ for a mixture of polar diaminotriazine herbicides (I), where $-X$ represents either $-Cl$ or



(I)

$-SCH_3$, and R is variously C_2, i-C_3, or t-C_4. In this case there is a single, polar entity (diaminotriazine) in each solute molecule, with differing numbers and kinds of various nonpolar or weakly polar

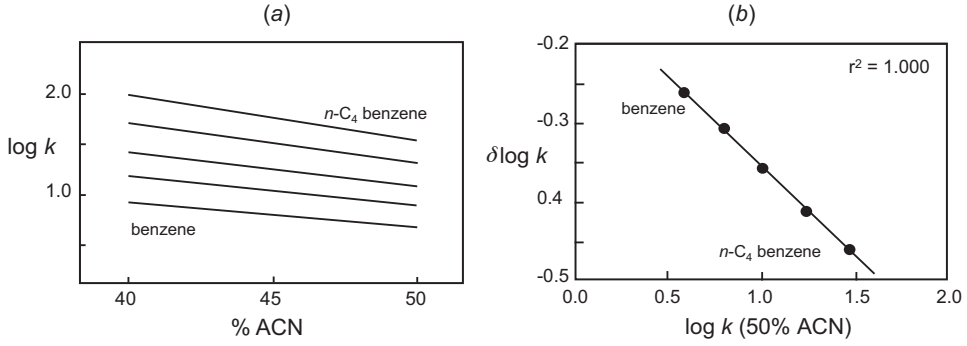


Fig. 1. Illustration of "regular" retention behavior for *n*-alkylbenzene solutes (#1–3, 5, 6 of Table 1). (a) plots of $\log k$ vs. %-ACN; (b) correlation of values of $\delta \log k$ (for +10% change in ACN concentration) with $\log k$ for 50% ACN and 35 °C. Data of [4,5]; see text for details.

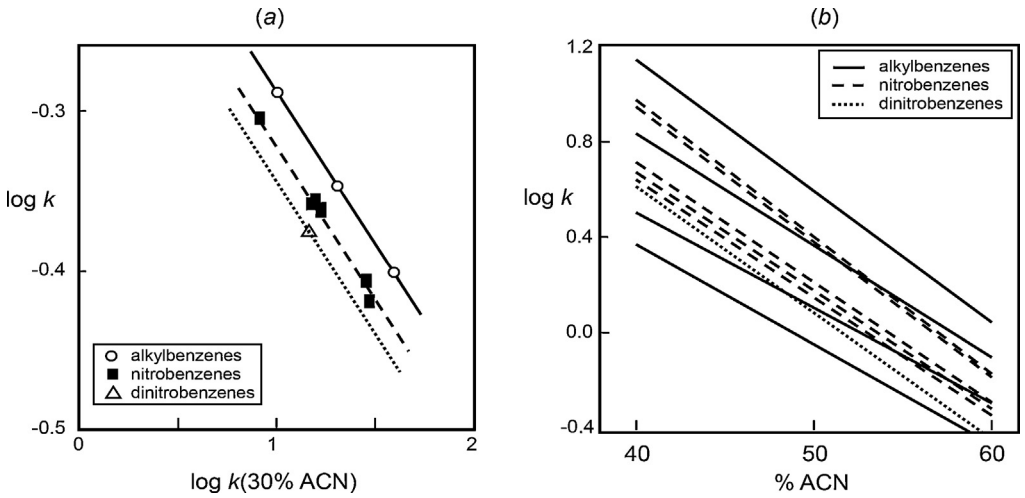
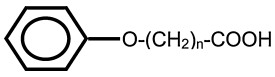


Fig. 2. An example of "non-regular" retention behavior for a mixture of nitro-substituted aromatics ((○) benzene, toluene, *m*-xylene; (●) nitrobenzene, 2-, 3- and 4-nitrotoluene, 2- and 4-nitro,1,3-xylene; (△) 2,6-dinitrotoluene). (a) Plot of $\delta \log k$ for a +10% change in ACN concentration vs. $\log k$ for 30%-ACN and 35 °C; (b) plots of $\log k$ vs. %-ACN for same solutes. Data of [6]; see text for details.

substituents. This sample clearly exhibits "regular" behavior. A similar result can be seen in Fig. 2a for molecules that contain a single $-NO_2$ group.

A second example in Fig. 3b shows a $\delta \log k$–$\log k$ plot for a mixture of phenoxycarboxylic acid herbicides (II) where the phenyl group is substituted by one or



(II)

more weakly polar groups (—Cl or $-CH_3$) in the 2-, 4-, and/or 5-positions, and n varies from one to three. There is a poorer correlation ($r^2 = 0.73$) in Fig. 3b, which is believed to be due to the presence of an ionizable —COOH group in each solute molecule. As we will shortly see, a change in %B (as in Fig. 3b) results in a change in mobile phase pH, which can further affect retention due to changes in solute ionization. When the compounds of Fig. 3b are replaced by their non-ionizable methyl esters (data of [7], not shown), ionization is no longer possible, and the correlation improves from $r^2 = 0.73$ to $r^2 = 0.97$; i.e., from non-"regular" to "regular" behavior. Figs. 2 and 3 show that mixtures of polar solute molecules can behave as "regular" samples when the same polar group(s) is present in all solute molecules, and there is no variable ionization of the polar group(s) in different sample molecules.

When a single substituent is replaced by different polar groups, the resulting compounds may approximate "regular" behavior. This is illustrated in Fig. 4 for a mixture of mono-substituted benzenes. Semi-parallel, non-crossing plots are observed in Fig. 4a for $40\% \leq \%B \leq 50\%$, and a linear plot of $\delta \log k$ vs. $\log k$ is observed in Fig. 4b. Values of k in RPC decrease for more "polar" solutes, so it can be concluded that values of $\delta \log k$ for an increase in %B become more negative as solute polarity increases (Fig. 4b). We can approximate the "polarity" of a substituent group in RPC by its negative contribution to $\log k$ (note the values of $\log k$ in Fig. 4b); some moderately "polar" aromatic substituents such as —Cl, —Br, and $-SCH_3$ appear effectively "nonpolar" in RPC, so far as their effect on "regular" behavior.

Certain conclusions can be drawn from the examples of Figs. 1–4:

- Values of $\delta \log k$ are a composite of three contributions.
- Changes in α with a change in %B require a difference in either polar substitution or ionization.
- "Regular" retention behavior is not limited to homologs.

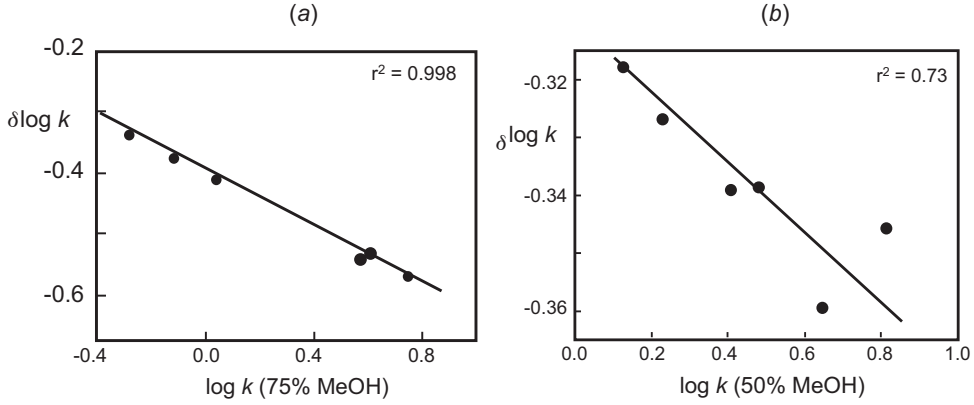


Fig. 3. Retention behavior for two different samples, each with a common polar entity as part of the sample molecules ($\delta \log k$ vs. $\log k$ plots for a change of %B). (a) Diaminotriazine herbicides, (b) phenoxycarboxylic acid herbicides (0.5 M acetate buffer (pH 2.9)). Data of [7]; see text for details.

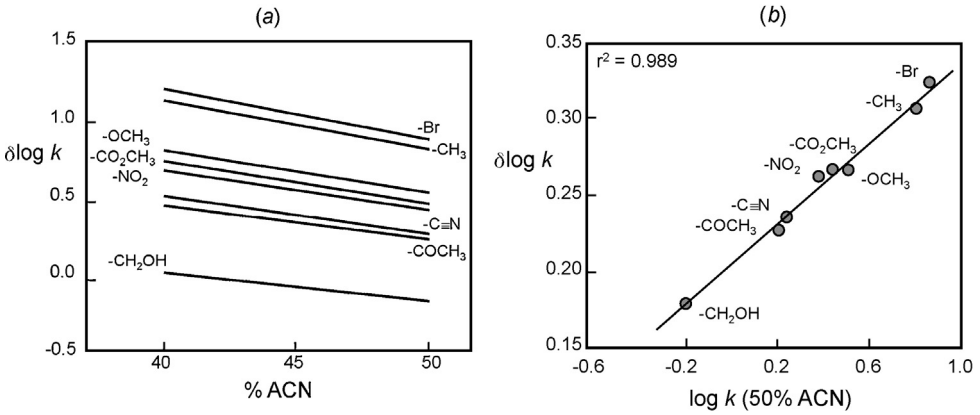


Fig. 4. "Regular" retention behavior for substituted benzenes of varying polarity (#2, 11, 13, 17, 18, 28, 29, 31 of Table 1); (a) plots of log k vs. %-ACN; (b) plot of $\delta \log k$ for a +10% change in ACN concentration vs. log k for 50% ACN; 35 °C. Data of [4,5]; see text for details.

First, when %B is changed, resulting values of $\delta \log k$ are a composite of three contributions: (a) "regular" retention behavior (e.g., Fig. 1b), (b) differences in the number and kind of polar substituents in the solute molecule (e.g., Fig. 2a), and (c) changes in solute ionization as a result of attendant changes in mobile phase pH (e.g., Fig. 3b).

Second, changes in relative retention (i.e., selectivity) occur only when latter factors b or c are operative; this in turn requires that for α to change significantly from an initial value near 1.0 as a result of a change in %B, the two compounds must differ in either polar substitution or ionization.

Third, "regular" behavior for a sample can occur whenever (a) polar groups are absent from sample molecules (e.g., nonpolar compounds such as alkyl- and halogen-substituted benzenes), (b) any polar part of the sample molecule remains the same (e.g., the diaminotriazine group I), or (c) *only* a single polar group X in the solute molecule varies (e.g., benzene substituted by X as in Fig. 4). However if the retention of a polar group is affected by adjacent nonpolar substituents for some (but not all) sample molecules, or if varying solute ionization occurs as a result of nonpolar substitution, sample "regularity" can be compromised.

In following Section 3.2, we will expand the concept of "regular" samples to include changes other than %B, namely temperature and the choice of organic solvent B. We note in passing that most samples are likely to be "non-regular," with moderate to major changes in selectivity as %B changes. On the other hand, two components of a sample might exhibit both similar retention and "regular" behavior, with the result that their separation cannot be much improved by a change of %B, temperature or organic solvent B. Isomeric solute pairs would seem likely candidates for such "regular" behavior, although in one study a simultaneous change of temperature and %B was found effective in achieving the separation of 124 of 137 such isomer pairs with resolution, $R_s > 1$ (for a column plate number $N = 15{,}000$) [8]. All of the 2145 compound-pairs of Table 1 ($66 + 65 + \cdots + 2 + 1$) can similarly be resolved with changes in temperature and %B, including seven isomer pairs (#3/4, 17/18, 23/24, 33/34, 35/36, 37/38, 64/65).

The preceding discussion of "regular" retention behavior is primarily useful as a basis for a closer examination of "non-regular" behavior and RPC selectivity (Section 3.2).

3.2. Changes in selectivity as a result of a change in temperature or mobile phase composition

The concept of "regular" retention for a change in %B can be extended to a change of temperature or the partial replacement of ACN by MeOH as B-solvent, as illustrated for five *n*-alkylbenzenes (○) and a change of temperature (Fig. 5a) or partial replacement of ACN by MeOH (Fig. 5b). This is also the case for homologs that contain a common polar substituent, as illustrated in Fig. 5c for three *n*-alkyl anilines, or Fig. 5d for three *n*-alkyl benzoic acids. In each of Fig. 5c and d, plots are shown for changes in temperature (□) or %-ACN (●), or the partial replacement of ACN by MeOH (▲).

It was noted above that values of $\delta \log k$ are the result of (a) contributions from "regular" retention, (b) differences in the number and kind of polar substituents in the solute molecule, and (c) changes in solute ionization. Only the latter two contributions to $\delta \log k$ (b and c) result in changed selectivity when conditions change, suggesting a closer look at these "non-regular" contributions to retention. In Fig. 5a a polar solute *trans*-4-nitro-chalcone (indicated by ■) deviates noticeably from this $\delta \log k$–$\log k$ correlation for the five alkylbenzenes (○); i.e., there is a significant departure from "regular" behavior for this solute.

Deviations $\delta\delta \log k$ from "regular" behavior (as in Fig. 5a for this polar solute) can be regarded as values of $\delta \log k$ that have been corrected for non-polar (or "regular") contributions to retention. That is, values of $\delta\delta \log k$ represent the "non-regular" portion of $\delta \log k$ resulting primarily from (a) differences in the number and kind of polar substituents in the solute molecule, and (b) changes in solute ionization. Values of $\delta\delta \log k$ should allow a better understanding of changes in relative retention (i.e., "selectivity") as a function of changed conditions. It will prove informative to explore the dependence of values of $\delta\delta \log k$ (and selectivity) on both the solute and separation conditions.

3.2.1. Changes in mobile phase pH as a result of changes in other conditions

Changes in mobile phase pH have been reported, e.g., [9–11], for a change in either %B or temperature. For a change in either temperature or pH, Dolan noted almost identical changes in selectivity for a mixture of acids and bases [12], while Lewis et al. [13] observed similar resolution maps for a mixture of anilines when either %-ACN or pH was varied. Changes in mobile phase pH can be inferred from changes in the retention of ionizable solutes, as in the two latter examples; these will be referred to as changes in "effective pH." The term "effective pH" recognizes that solute pK_a values and mobile phase pH are together affected by a change in conditions [1]; "effective pH" will be distinguished here from the pH of the buffer. The potential magnitude of these changes in "effective pH" is of practical interest, as this can influence our approach to optimize selectivity for samples that contain ionizable compounds.

Values of $\delta \log k$ for various anilines, pyridines, and benzoic acids (#51–67 of Table 1) have been reported [5] for a change in temperature or %B, or the partial replacement of ACN by MeOH. Values of

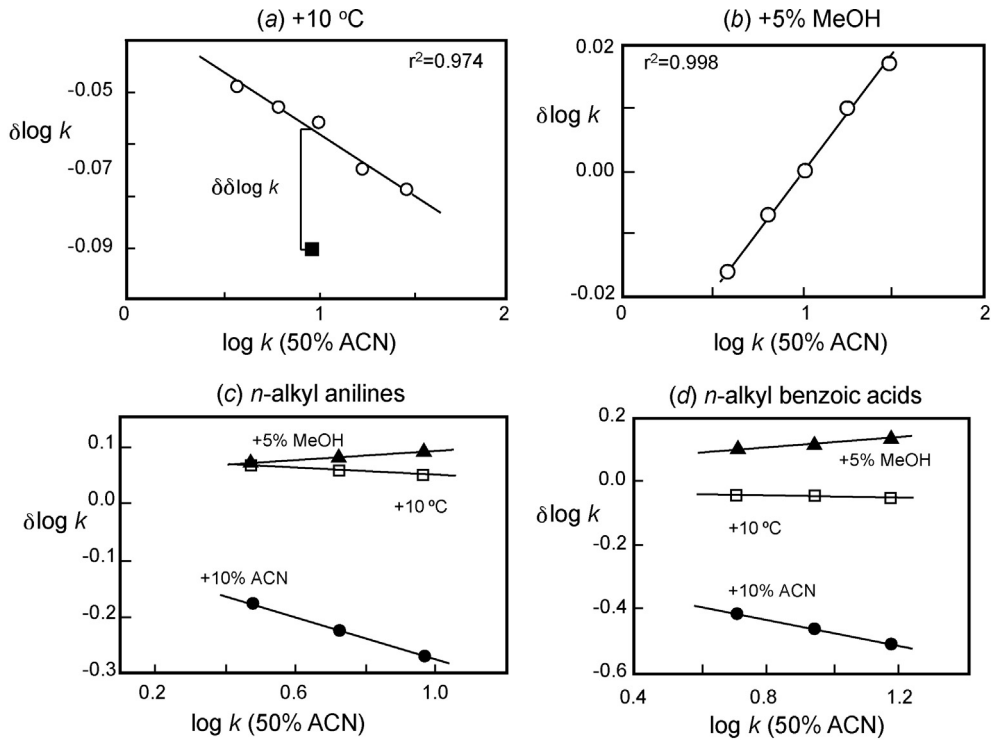


Fig. 5. Examples of "regular" retention behavior when different conditions are changed. Plots of $\delta \log k$ vs. $\log k$ for five *n*-alkylbenzenes (#1–3, 5, 6 of Table 1) and (a) a +10 °C change in temperature or (b) replacement of 5% ACN by 5% MeOH. Similar plots for *p*-substituted *n*-alkyl anilines (#51–53) (c), or *n*-alkyl benzoic acids (#60–62) (d), for change in temperature (□) or %-ACN (●), or partial replacement of ACN by MeOH (▲); $\log k$ values for 50%-ACN, 35 °C. Data of [4,5]; see text for details.

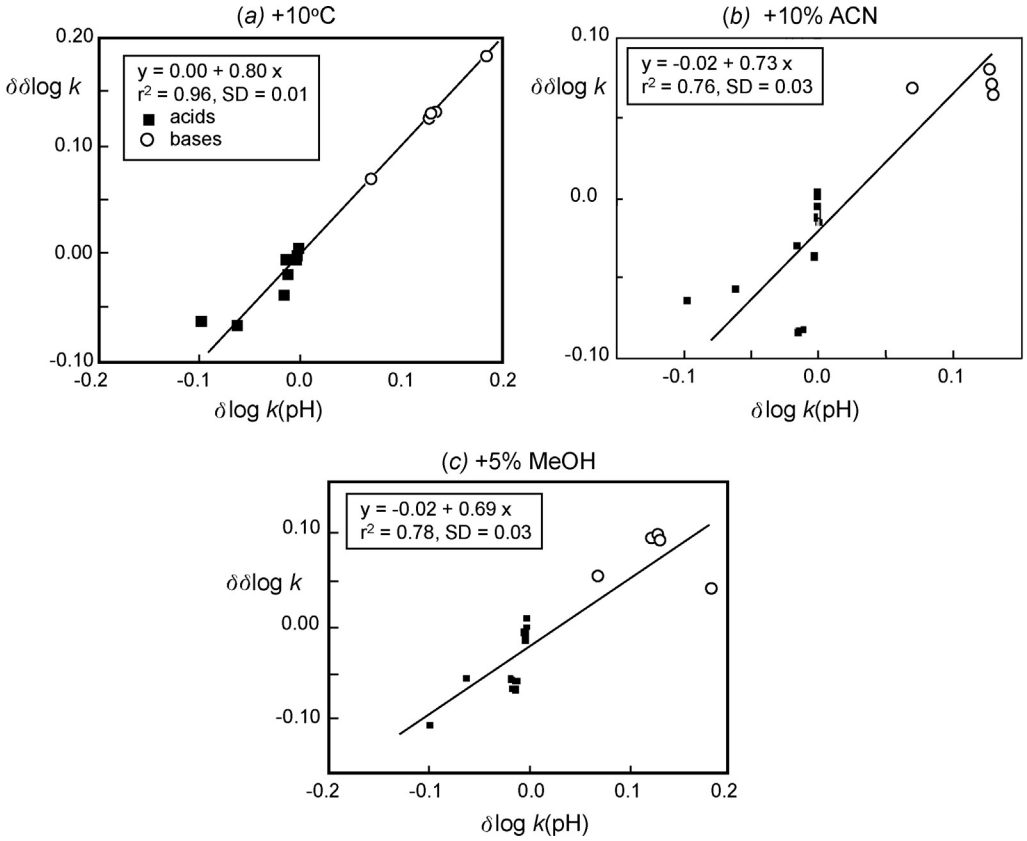


Fig. 6. Change of "effective pH" with a change in temperature (a) or %-ACN (b), or partial substitution of MeOH for ACN as B-solvent (c). Sample is a mixture of partly-ionized benzoic acids, anilines and pyridines (#51–67 of Table 1, data of [5]). In order to compensate for the contribution of a polar —COOH group to values of $\delta\delta \log k$ in Fig. 6 (see Appendix A), values of $\delta\delta \log k$ for the benzoic acids are relative to average values of $\delta\delta \log k$ for three non-ionized *n*-alkylbenzoic acids. Similar corrections for other polar substituents was generally not feasible, which presumably accounts for much of the scatter of data. See text for details.

Table 2
Relative effectiveness of a change of conditions for a change in selectivity (average values of $|\delta\delta \log k|$); based on data of [5].

	Avg. $\|\delta\delta \log k\|$		
	Neutral[b]	Ionizable[c]	Cations[d]
+40 °C	0.04	0.18	0.08
+30% ACN	0.23	0.37	1.12
50% MeOH[a]	0.21	0.79	1.90

[a] Complete replacement of ACN by MeOH to give 50% MeOH/buffer.
[b] Compounds #1–45 or Table 1.
[c] Compounds #51–67 of Table 1.
[d] Compounds #46–50 of Table 1 (completely ionized strong bases).

$\delta\delta \log k$ for the latter compounds are plotted in Fig. 6 for a change of temperature (Fig. 6a) or %B (Fig. 6b), or partial replacement of ACN by MeOH (Fig. 6c), in each case vs. values of $\delta \log k$ for a change of buffer pH ($\delta \log k$(pH)) by +0.2 units.

If changes in $\delta\delta \log k$ for some change in conditions (e.g., +10 °C in Fig. 6a) correlate strongly with $\delta \log k$ for some other change in conditions (e.g., pH in Fig. 6a), changes in the two conditions (e.g., temperature and pH) have an equivalent effect on changing selectivity for that sample. Such knowledge can be useful when prioritizing the order in which to examine different variables during high performance liquid chromatography (HPLC) optimization. For example, if temperature and pH are expected to have similar results in terms of changing selectivity for a sample, one variable could be examined early in the process and the other not examined or its use delayed until other, more promising, variables had been examined. Furthermore, if two variables show correlated behavior, the one that is most convenient to manipulate (e.g., temperature) could be examined first and the less convenient variable (e.g., pH) left to later, or not examined at all.

The similar changes in $\delta\delta \log k$ in Fig. 6a–c suggest that changes in temperature, %-ACN, or MeOH substitution all mimic a change in mobile phase pH. Significant correlations ($0.76 \le r^2 < 0.96$) are observed in each of Fig. 6a–c, with the slope of each plot being proportional to the corresponding change in "effective pH" of the mobile phase. Scatter in these plots appears due mainly to the presence of other polar substituents in these solute molecules, which can also affect values of $\delta\delta \log k$ (see Appendix A).

3.2.1.1. Achievable increases in selectivity. During selectivity optimization, a variation of temperature over a wider range (e.g., a range of 40 °C vs. 10 °C) increases the probability of finding a more favorable peak spacing and better resolution. Corresponding changes in $\delta\delta \log k$ will be approximately proportional to the relative change in temperature (e.g., 4-fold larger for a change from +10 to +40 °C). The maximum range over which conditions can be changed will be limited by various practical considerations; here we will arbitrarily assume maximum changes of 40 °C, 30% B, and a complete replacement of ACN by MeOH (in the present case, 50% ACN-buffer replaced by 50% MeOH-buffer). Thus relative to the changes in conditions described in the Section 2 ($\delta \log k$ values for +10 °C, +10% B, and +5% MeOH in the B-solvent), values of $\delta\delta \log k$ shown in Fig. 6 can be increased by 4-, 3-, and (more approximately) 10-fold, respectively. Resulting average values of $|\delta\delta \log k|$ for 45 neutral, 17 ionizable, and five cationic solutes are shown in Table 2. The latter values provide a relative measure of the effectiveness of each change in condition for improving selectivity and resolution, as a function of sample type.

Values of $|\delta\delta \log k|$ for ionic samples are generally larger, suggesting that such samples are more easily separated when varying any of the three conditions of Table 2. This was seen also in a previous study [14] for several samples when temperature or %B was varied. Larger values of $|\delta\delta \log k|$ for ionizable samples are partly the result of changes in "effective pH," and partly due to associated changes in the column (note the values of $|\delta\delta \log k|$ for completely ionized cations in Table 2).

A change of temperature is seen in Table 2 to be relatively ineffectual for neutral samples; it's main effect on selectivity appears to be a change in "effective pH." For a +40 °C change, the resulting change in "effective pH" can be estimated from the slope of the plot of Fig. 6a (+0.80). The latter value is for a change in temperature of only +10 °C, so for a +40 °C change in temperature the slope should increase to about $4 \times 0.8 = 3.2$. However the axis of Fig. 6a–c ($\delta \log k$(pH)) corresponds to a change in buffer pH of only +0.2 units, so the resulting change in "effective pH" for a temperature change of +40 °C can be estimated as $0.2 \times 3.4 = 0.64$ units. A corresponding value of 0.25 units was observed in the study of Dolan [12] for an increase in temperature of +20 °C, which corresponds to 0.5 units for +40 °C (in approximate agreement with the preceding value of 0.64). Changes in "effective pH" for a 30% change in the organic solvent (or a 10-fold change in gradient time) can similarly be estimated at 0.44 units, which compares with a value of 0.5–0.7 units from the study of [13], for the same 30%B change. Finally, for a complete change of ACN by MeOH, there is an increase in "effective pH" of about 1.4 units These changes in pH are large enough to suggest that *simultaneous* changes in temperature, %-organic, and organic solvent can mimic changes in buffer pH by as much as 1–2 units, thus making the variation of buffer pH somewhat redundant and less useful as a means of controlling selectivity.

3.2.2. Column selectivity

The selectivity of different columns can be compared by means of the hydrophobic-subtraction (H-S) model of RPC column selectivity [15,16]. Values of k for a column can be described by certain solute characteristics and column properties:

$$\log k = \log k_{EB} + \eta' H + \sigma' S^* + \beta' A + \alpha' B + \kappa' C \quad (6)$$

Values of k for different compounds can be related to k for a reference compound (ethylbenzene, k_{EB}) and five terms that refer to different solute-column interactions. Solute properties are represented by η' (hydrophobicity), σ' (molecular "bulkiness"), β' (hydrogen-bond (H-B) basicity), α' (H-B acidity) and κ' (approximate charge on the solute molecule). The column parameters H (column hydrophobicity), S^* (resistance in the stationary phase to solute penetration), A (column hydrogen-bond acidity), B (column hydrogen-bond basicity), and C (column ion-exchange capacity; specifically, the relative negative charge in the stationary phase) are complementary properties of the column, values of which are available for about 600 different columns [17]. The difference in selectivity of two columns *1* and *2* can be measured by the function F_s [16]:

$$F_s = \{[12.5(H_2 - H_1)]^2 + [100(S_2^* - S_1^*)]^2 + [30(A_2 - A_1)]^2 + [143(B_2 - B_1)]^2 + [83(C_2 - C_1)]^2\}^{1/2} \quad (7)$$

where H_1 and H_2 refer to values of H for columns *1* and *2*, and similarly for values of S^*, A, etc. The largest change in selectivity for two columns that have so far been characterized by means of Eq. (6) (i.e., with measured values of H, S^*, etc.) corresponds to a value of $F_s \approx 300$.

A change in conditions other than the column might affect selectivity in similar fashion as a change of column. This possibility can be evaluated in the same manner as the development of Eq. (6), by carrying out a regression of values of $\delta\delta \log k$ for a given change in condition vs. values of the solute parameters η', σ', etc.:

$$\delta\delta \log k = \delta H \eta' + \delta S^* \sigma + \delta A \beta' + \delta B \alpha' + \delta C \kappa' \quad (8)$$

Here δH represents the equivalent change in column hydrophobicity H as a result of some change in another condition, and

similarly for δS^*, δA, etc. In order to avoid the complication of change in "equivalent pH," the latter regression was restricted to neutral solutes #1–45 of Table 1 and related values of η', σ', etc.).

Table 3 summarizes regressions according to Eq. (7) for "maximum" changes in temperature and %B, and complete replacement of ACN by MeOH. The calculated change in values of H, S^*, etc. (i.e., δH, δS^*, etc.) as a result of a change in temperature, %B, or replacement of ACN by MeOH can be compared with typical changes in values of H, S^*, etc. for columns of different type (alkyl, phenyl, cyano, etc.; last column of Table 3). It is seen that simultaneous changes in the three conditions of Table 3 are able to create generally comparable changes in selectivity as a change of column, but in *continuous* fashion as opposed to a simple change of column (a discrete, non-continuous change in selectivity). Furthermore, no single column allows maximum changes in all five column parameters. Thus a change in column seems unlikely to offer as useful a change in selectivity, compared to that produced by a combination of feasible changes in temperature, %B, and/or the ratio of MeOH and ACN in the mobile phase. Finally, maximum changes in the three conditions of Table 3 correspond to a value of $F_s = 280$ (for $\delta H = 0.48$, $\delta S^* = 0.57$, $\delta A = 0.23$, $\delta B = 1.26$, and $\delta C = 2.47$). (Note that the latter values of δH, δS^*, etc. correspond to the *range* of values in Table 3 for individual conditions.) This value of $F_s = 280$ is comparable to that found for a maximum change of column selectivity ($F_s \approx 300$, [17]).

The preceding argument for a duplication of column selectivity by changes in temperature, %B, and replacement of ACN by MeOH must be qualified to some extent. The column parameters H, S^*, etc. do not take into account two additional interactions between the solute and column: π–π interaction and dipole–dipole [1,16] (although the number of solutes and columns that are significantly affected by these effects is relatively small). It should also be pointed out that the correlations of Table 3 are significantly less than $r^2 = 1$, and the standard errors associated with these correlations are fairly large (0.05–0.20); this suggests that some aspects of column selectivity may *not* be replicable by changes in other conditions. Finally, a change of column may offer advantages other than a change of selectivity [18]; for example, improved peak shape and ruggedness, increased column stability, and better column-to-column reproducibility.

3.3. Strategies for selectivity optimization

Selectivity can be improved by

- variation of %B (or gradient time)
- variation of B-solvent (ACN, MeOH)
- variation of temperature
- variation of pH
- change of column

Note that continuously "varying" a condition allows a much greater control over selectivity than does a discrete "change" of condition (e.g., Fig. 2b). The column can only be changed, not continuously varied. Certain additional conditions can also be used to vary selectivity (e.g., buffer type and concentration, ion-pairing) but for various reasons are used only infrequently in RPC.

It is now accepted that an effective approach to controlling selectivity and maximizing resolution consists of simultaneous varying two or more conditions (e.g., [2,3]). Presently available software [3] allows for computer simulation where as many as three different "selectivity" conditions are simultaneously varied. One such experimental design is shown for the gradient experiments of Fig. 7,

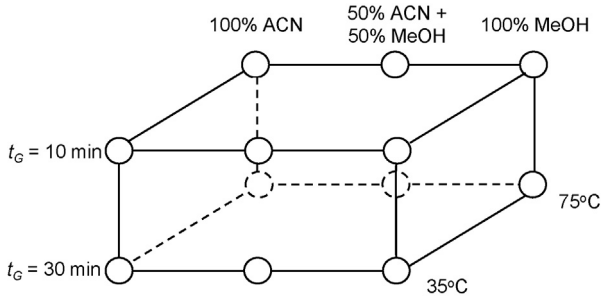


Fig. 7. Experimental design for the simultaneous optimization of temperature, gradient time, and mixtures of ACN and MeOH. Gradient times can be varied for different samples or other conditions; see [1] for further information.

where gradient time t_G, column temperature, and the ACN/MeOH ratio of the organic component of the mobile phase are varied. Variation of pH can be substituted for any of the three conditions of Fig. 7, but Section 3.2.1 argues that this may be a less useful alternative. Note that a change of t_G by 3-fold (as in Fig. 7) is approximately equivalent to a change in %B by 10% [1] (computer simulation allows extrapolation for larger and smaller gradient times, comparable to an isocratic change of 30%B). Similarly, values of $\delta\delta \log k$ measured isocratically (as in the present study) have the same significance for gradient elution [19]. That is, for corresponding isocratic and gradient conditions, values of k are the same for a given solute.

When the approach of Fig. 7 proves disappointing, the gradient experiments of Fig. 7 can be repeated with a change of either buffer pH or column. Changes in the conditions of Fig. 7 result in changes in "effective pH" of one to two units, but an optimum pH may lie more than 2 units away from the pH of the initial buffer. Before carrying out the experiments of Fig. 7, therefore, it may prove useful to assess the approximate effect of pH on separation. This can be achieved with a few survey experiments where only pH is changed; e.g., pH = 2.5, 4.0, 5.5, and 7.0. A pH which approximates the pKa values of critical solutes will generally be preferred for a maximum influence of changes in pH on selectivity, and this can be recognized by observing peak movement as a function of pH [1]. When the buffer pH $\approx$ sample pK_a, changes in retention and selectivity will be most apparent for a given change in pH; the experimental design of Fig. 7 can then follow at this buffer pH. Alternatively, a knowledge of the structures of sample solutes may allow rough estimates of solute pK_a values and a preferred buffer pH [1].

It should also be noted that a method that operates near the pK_a of one or more solutes, is likely to be less robust. The small changes in pH that are effective for fine-tuning selectivity are also the small changes in "effective pH" that may occur from normal (unintended) variations in temperature, pH, and/or solvent ratio during routine operation. For this reason, it often is desirable to chose a mobile phase pH that is >1.5–2 pH units away from the pK_a of critical solutes, so as to avoid undesirable changes in "effective pH" due to normal method variability. This further suggests that pH should have a lower priority than other variables when optimizing selectivity, although it should be noted that the present recommendations are based on results for just one buffer (phosphate at low pH). The extension to other buffers is certainly not quantitative, and for some buffers effective pH can change in the opposite direction with a change of %B. For further details, see [20].

A change of column as a means of changing selectivity appears less promising, in view of the apparent duplication by other conditions of changes in selectivity when the column is changed

Table 3
Equivalent changes in column selectivity properties (H, S^*, etc.) from the indicated changes in other conditions (Eq. (8)).

	Regression results (Eq. (8))[a]			Maximum range in H, S^*, etc.[c]
	+40 °C	+30% ACN	50% MeOH[b]	
r^2	0.666	0.942	0.904	
Std. error	0.046	0.078	0.195	
Intercept (forced)	0.000	0.000	0.000	
δH	−0.003	0.274	−0.204	0.64
δS^*	0.007	−0.263	0.307	0.14
δA	0.225	0.014	0.087	0.74
δB	−0.005	−0.338	0.921	0.14
δC	0.071	−0.770	1.701	1.74

[a] Based on $\delta \log k$ values of [5], excluding partly-ionized compounds #51–55,59, 63–67 of Table 1).
[b] For a mobile phase of 50% MeOH/buffer.
[c] Average values for RPC columns of different type; data of Table 5.8a of [1], excluding type-A columns.

(Table 3). However an initial exploration of different columns may be worthwhile (prior to changing other conditions), in order to anticipate potential problems with peak tailing or poor retention for very polar components. Another advantage of the initial use of more than one column is if two (or more) components of the sample overlap *and* subsequently exhibit "regular" behavior. In such cases, if one of the columns initially studied was able to separate these compounds, that column might prove a better choice in combination with the further optimization of other conditions. When carrying out column screening in this way, the columns should be selected to be as different as possible in terms of selectivity [16,17].

The latter recommendations can be summarized as follows:

1. Select buffer pH (usually 2 < pH < 4).
2. Trial gradient separations on 2–4 columns of different selectivity (large F_s); select one column for further experiments.
3. Carry out gradient separations for two different temperatures and two gradient times (Fig. 7); select conditions for optimum resolution.
4. If resolution and/or gradient time are unacceptable, complete remaining 8 separations of Fig. 7 (different temperatures, gradient times, and organic solvent).
5. If resolution and/or gradient time are still unacceptable:

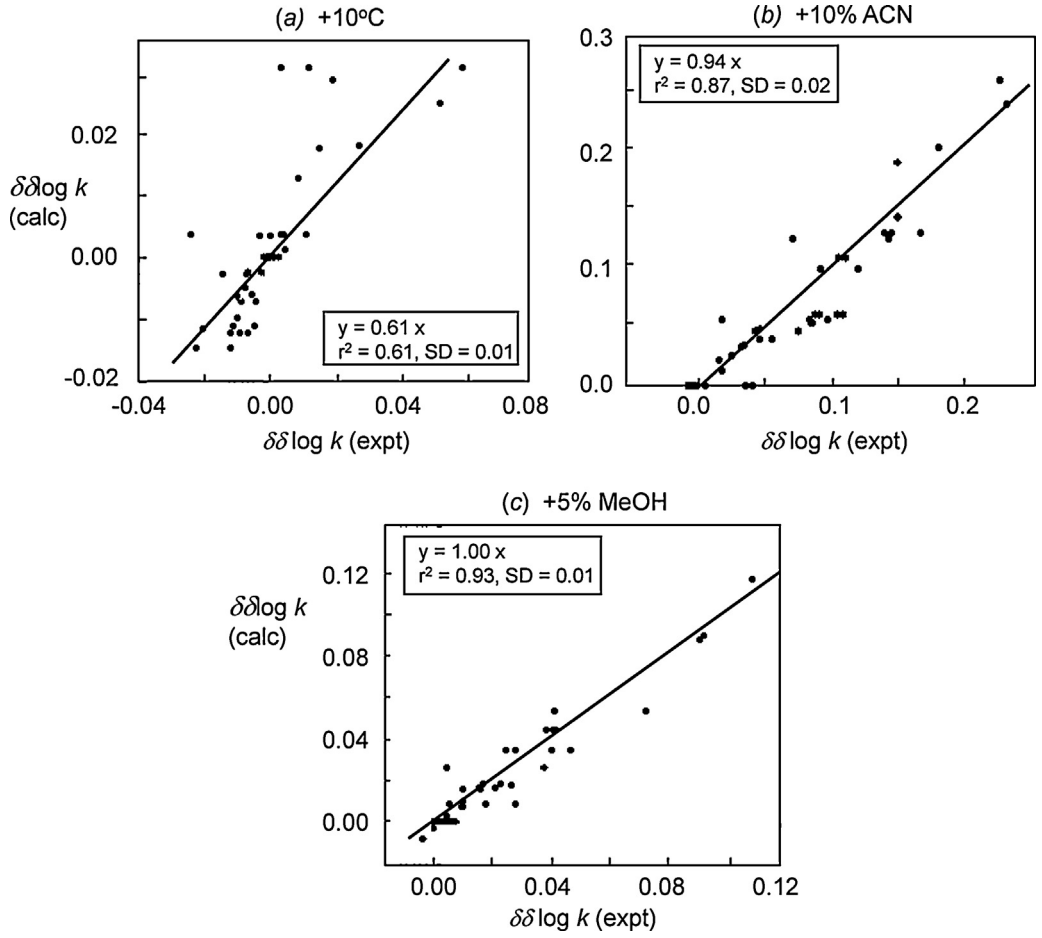


Fig. 8. Comparison of values of $\delta\delta \log k$ for neutral solutes (#1–45 of Table 1) calculated from Eq. (8) vs. experimental values for a change in temperature (a) or %-ACN (b), or partial replacement of ACN by MeOH (c).

Table 4
Correlation of values of $\delta\delta \log k$ with solute polar groups (Eq. (8)); neutral solutes #1–45 of Table 1.

	Regression results (Eq. (8))		
	+10 °C	+10% ACN	45/5% ACN/MeOH
r^2	0.612	0.844	0.927
SE	0.011	0.027	0.008
a (amide)	0.031	−0.131	0.044
b (alcohol)	0.004	−0.060	0.034
c (phenol)	−0.003	−0.056	0.009
d (keto)	0.003	−0.040	0.007
e (nitrile)	−0.010	−0.049	−0.003
g (nitro)	−0.012	−0.047	0.000
h (ester)	−0.004	−0.034	0.009
i (ether)	−0.005	−0.056	0.003
j (aromatic rings[a])	−0.006	−0.013	0.009

[a] Effect on $\delta\delta \log k$ of each aromatic ring in the solute molecule.

a. change the column if one pair of compounds is always unresolved, but was resolved with a different column from step-2
b. change buffer pH by >2 units and repeat steps 3 and 4
c. consider other means of changing selectivity ("special" column, or some other change in conditions)

However it must be emphasized that selectivity optimization is a complex process which may benefit from different approaches for different samples. The present recommendations, which are of a general nature, may therefore prove more applicable in some cases, and less so in others.

4. Conclusions

Changes in reversed-phase retention ($\delta \log k$) have been examined as a function of the sample and changes in the following conditions:

1. temperature
2. concentration of organic solvent in the mobile phase (%B)
3. relative concentrations of acetonitrile and methanol in the mobile phase
4. buffer pH

Values of $\delta \log k$ are affected by both nonpolar and polar interactions, while the latter play the major role in determining selectivity as a function of conditions. The contribution ($\delta\delta \log k$) of polar interactions to values of $\delta \log k$ can be determined, and values of $\delta\delta \log k$ have been used to assess the relative usefulness of a change in different conditions during selectivity optimization. It was found that changes in each of conditions 1–3 above replicate, to some extent, a change in either buffer pH or the column. This brings into question the relative value of varying buffer pH or a change of column as a means of further improvements in selectivity and resolution, compared to (or in addition to) simultaneous changes in two or more of conditions 1–3 above. However, initial exploratory experiments where pH and/or the column are changed can be recommended for various reasons.

For the separation of challenging samples by reversed-phase HPLC, the simultaneous variation of two or more conditions that affect selectivity has been recommended, followed by the use of computer simulation to determine conditions for maximum resolution [1]. The present study suggests the similar use of conditions 1–3 in this way (see Fig. 7), in some cases supplemented with an initial survey of the effect on peak spacing of large changes in buffer pH, and/or a similar survey of separations with different columns.

The present analysis also examines so-called "regular" samples, whose selectivity is little affected by a change in conditions. Some such samples might therefore prove difficult to separate, although their occurrence in the average laboratory seems somewhat less likely. An initial column screen offers a possible solution to the latter problem when it arises.

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Appendix A. Dependence of values of $\delta\delta \log k$ on polar groups within the solute molecule

The present study suggests that values of $\delta\delta \log k$ are related to the number and kinds of polar groups within the solute molecule. A further examination of this relationship is best confined to non-ionizable ("neutral") compounds, because this avoids the complication of changes in "effective pH" when conditions are changed. Values of $\delta \log k$ for 45 neutral compounds are reported in [5]. It might be anticipated that $\delta\delta \log k$ should correlate with the number *n* and kind of polar substituents *i* for each solute; i.e.,

$$\delta\delta \log k = \sum nf(i) \tag{9}$$

where $f(i)$ is a measure of the polarity of group *i*. More polar substituents in solutes #1–45 of Table 1 include the following functional groups: amide (—CONH—), alcohol (R-OH), phenol (Ar-OH), keto (>C=O), nitrile (—C≡N), nitro ($—NO_2$), ester ($—CO_2—$), and ether (—O—), as well as the number of additional aromatic rings in the molecule (n_{Ar}; e.g., equal 0 for aliphatic solutes, +1 for substituted benzenes, etc.). The regression of values of $\delta\delta \log k$ vs. the number of each polar group in the solute molecule was next investigated for each change of condition:

$$\delta\delta \log k = an_{CONH} + bn_{ROH} + cn_{Ar\text{–}OH} + dn_{C=O} + en_{CN} + fn_{NO_2} + gn_{CO_2} + hn_{O} + in_{Ar} \tag{10}$$

Here, values of *a*, *b*, etc. are proportional to substituent group polarity values $f(i)$, while n_i refers to the number of groups *i* of a given kind (amide, alcohol, etc.). Regressions for each change of condition are summarized in Table 4. Calculated values of $\delta\delta \log k$ from Eq. (10) are compared with experimental values in Fig. 8.

Reasonable correlations with Eq. (9) are noted in Table 4 for a change of % ACN ($r^2 = 0.84$) or substitution of ACN by MeOH ($r^2 = 0.93$), while a change of temperature is only moderately correlated ($r^2 = 0.61$). A likely cause for values of $r^2 < 1$ is variation in values of $f(i)$ for a given substituent *i* such as >C=O, due to changes in the polarity of *i* as a result of other substituents in the vicinity of *i*.

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