Journal of Chromatography, 592 (1992) 197–208
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2441

Computer simulation for the prediction of separation as a function of pH for reversed-phase high-performance liquid chromatography

II. Resolution as a function of simultaneous change in pH and solvent strength

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ABSTRACT

The optimization of reversed-phase high-performance liquid chromatographic separation by the simultaneous variation of pH and solvent strength (%B) was studied for acidic (substituted benzoic acids) and basic samples (substituted anilines). The combination of these two variables was expected to be more useful than either variable alone. This proved to be the case for the benzoic acid sample, but not for the aniline sample. Column plate numbers were also studied for each sample and as a function of pH. With the exception of one compound (3,5-dimethylaniline) in one particular pH range (3.0–4.5), plate numbers of 12 000–20 000 were observed for each sample.

INTRODUCTION

In Part I [1] we discussed the use of computer simulation (based on a theoretical model) for the prediction of high-performance liquid chromatography (HPLC) separation as a function of pH. It was shown that this approach to HPLC method development is reliable if (a) appropriate experimental data are selected as input to computer simulation and (b) predictions are restricted to a suitable range of mobile phase pH. HPLC method development based on selecting an optimum mobile phase pH is only effective for samples that contain acidic or basic solutes whose ionization and retention change as a function of pH. As retention (k') can decrease by a factor of ten or more for an ionized *vs.* a non-ionized compound, it is often necessary to combine pH optimization with variation in solvent strength (%B) in order to maintain a reasonable k' range for the resulting separation. Ideally, the retention of sample components in the final (optimized) separation should fall in the range $1 < k' < 20$ [2].

A few recent papers [3–5] have dealt with the combined variation of mobile phase pH and solvent strength; empirical functions for retention as a function of both pH and %B were used in conventional factorial-design procedures. Marques and Schoenmakers [6] provide a detailed study of several models for use in mapping the combined effects of pH and %B on sample retention.

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When solvent strength is varied apart from a change in mobile phase pH, significant changes in band spacing (values of α) can occur [7]. This may lead in turn to useful changes in sample resolution as a function of %B. We have shown previously that computer simulation of separation as a function of %B can be a valuable aid in HPLC method development [7,8]. Simultaneous changes in both pH and %B therefore seem likely to result in further improvements in separation for acidic and/or basic samples, compared with the case of changes in either %B or pH alone. This was in fact demonstrated by a preceding study of a sample of substituted benzoic acids [9,10].

In this paper we shall examine several experimental systems that involve simultaneous changes in mobile phase pH and %B. Changes in both retention and column efficiency as a function of these variables were studied. As a result, it was possible to draw some general conclusions on how sample resolution varies as a function of mobile phase pH and %B.

THEORY

A quantitative model for sample retention in reversed-phase HPLC as a function of pH was described in Part I [1]. Reversed-phase retention as a function of %B is much more complicated and difficult to model in terms of basic theory. The use of solubility-parameter theory leads to an expression of the form [11]

$$\log k' = A\varphi^2 + B\varphi + C \quad (1)$$

where A, B and C are constants for a given solute and mobile phase organic solvent and φ is the volume-fraction of organic solvent B in the mobile phase; $\varphi = 0.01$ (%B). The model on which eqn. 1 is based is overly simplistic, but actual experimental data are described by eqn. 1 fairly well [12]. For many HPLC systems, eqn. 1 can be simplified (with little error) to

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

Here $\log k_w$ is equal to C in eqn. 1, S is equal to $-B$ and the term $A\varphi^2$ is ignored. We have previously used eqn. 2 as a basis for computer simulations as a function of %B [8–10]. Our latest computer simulation software (DryLab I/mp) allows the use of either eqn. 1 or 2 as a basis for predicting separation as a function of %B. Eqn. 1 requires three initial HPLC runs where only %B is varied, whereas eqn. 2 requires only two runs.

Simultaneous variation of mobile phase %B and pH

In this study we used DryLab I/mp for the simultaneous optimization of both %B and pH, in order to maximize sample resolution. Although the present version of this software provides for variation of only one separation variable at a time, two-variable optimization can be achieved as follows. Three experimental runs at different pH values (*e.g.*, pH = 3, 4 and 5) are carried out for two different values of %B (*e.g.*, 25 and 35% methanol). Computer simulation can be used to predict retention as a function of pH for each %B value. Now the predicted retention times for any pH value can be used to predict separation as a function of %B. The end result is that six initial experimental runs allow the prediction of separation as a function of both pH and %B (based on eqn. 2). If three different %B values are selected instead (nine runs), more accurate predictions of separation as a function of %B and pH can be carried out (in the same way) based on eqn. 1. Later versions of DryLab I/mp will allow the more convenient, simultaneous variation of pH and %B (without re-entry of data for variation of %B).

EXPERIMENTAL

The equipment, materials and procedures used in this study were described in Part I [1]. Plate numbers are calculated by $N = 5.54\,(t_R/W_{1/2})^2$, where $W_{1/2}$ refers to the band width at half-height.

RESULTS AND DISCUSSION

Solute band widths

The primary goal of computer simulation is the estimation of resolution as a function of experimental conditions. This in turn requires predictions of both retention time and band width for each sample component as one or more conditions are varied during method development. In Part I [1] we discussed the prediction of resolution (values of α) as a function of mobile phase pH, while earlier papers addressed retention as a function of %B [9,12].

Band width can be used interchangably with column plate number, and a vast literature exists concerning plate number as a function of experimental conditions [13–15]. In many instances it is possible to predict plate number and band width as a function of solute structure and experimental conditions with reasonable accuracy [16]. Computer simulation based on DryLab software makes use of this prior theory. However, these predictions of plate number assume "ideal" chromatography, which is not always observed experimentally. Acidic and basic solutes are often found to deviate from "ideal" behavior [17,18], generally exhibiting wider (often tailing) bands than are predicted by theory. In this study we examined solute plate number as a function of experimental conditions for the acidic and basic solutes described in Part I [1].

Experimental band widths vs. values predicted by theory. Several dozen runs were carried out for the benzoic acid and aniline samples discussed in Part I [1] as a function of pH, %B and other variables. The resulting chromatograms were generally comparable in terms of band width and average plate number; calculated values of N for the various sample components in most cases fell within a range of N = 10 000–20 000, with average values of about 16 000. Fig. 1 shows a typical example: the separation of the substituted aniline sample with a mobile phase of pH = 3.00 and 25% B. Table I summarizes plate number measurements for all of the aniline bands as a function of pH.

DryLab I/mp can provide estimates of solute plate number as a function of solute molecular weight and experimental conditions; these values correspond to plate numbers expected for "ideal" chromatography. Fig. 2 shows two typical comparisons of experimental *vs.* predicted values of N as a function of solute retention time, t_R. The solid curve represents values predicted by theory (DryLab I/mp) and the open circles are experimental values. Data in Fig. 2A are taken from a separation of the substituted benzoic acid sample [10] and Fig. 2B is from a separation of the present aniline sample. There is reasonable agreement between experimental and predicted values of N in each instance[a], *i.e.*, the average deviation of predicted *vs.* experimental values is ±13% for the benzoic acid sample and ±23% for the substituted anilines. These deviations correspond to errors in resolution (and band width) of ±6 and ±11%, respectively. Other sep-

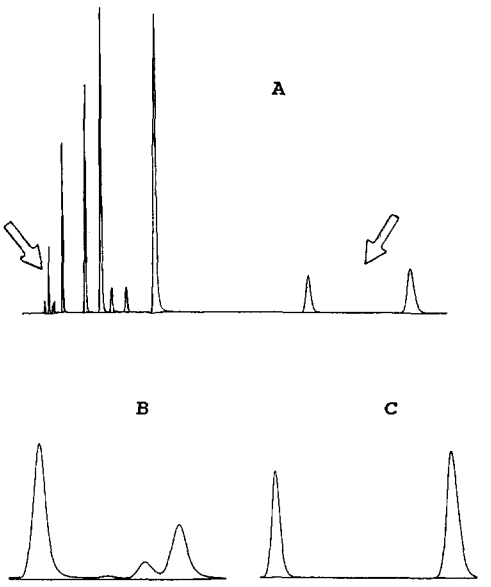

Fig. 1. Separation of substituted anilines sample. Conditions: 25 × 0.46 cm I.D. StableBond CN column; 25% methanol–buffer (25 m*M*, pH 3.00); 35°C; 1 ml/min. (A) Whole chromatogram; (B) enlargement of front of A (arrow); (C) enlargement of end of A (arrow).

TABLE I

AVERAGE PLATE NUMBERS FOR THE SUBSTITUTED ANILINE SAMPLE AS A FUNCTION OF MOBILE PHASE pH

Conditions: 25 × 0.46 cm I.D. StableBond CN column; 25% methanol–buffer (25 m*M*); 35°C; 1 ml/min.

pH	N[a]
2.00	15 800 ± 1300
3.00	16 200 ± 3300
4.00	16 100 ± 1600
6.00	16 300 ± 800
Average	16 100 ± 1800

[a] Average value for all solutes; excludes dimethylaniline (DMA) for 3 < pH < 4.5.

[a] Note the expanded y-axis in Fig. 2.

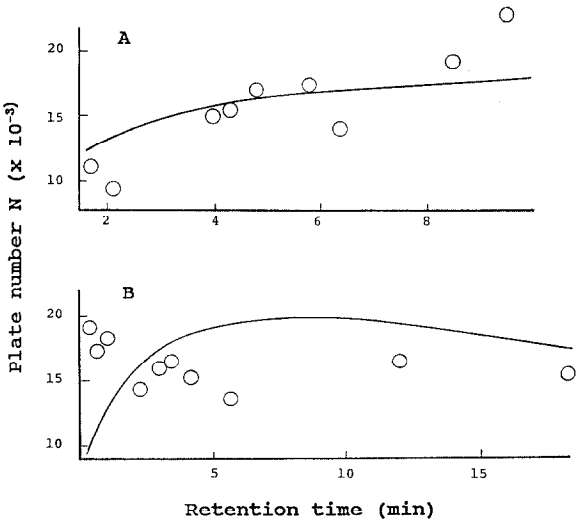

Fig. 2. Plate number as a function of retention time. (A) Benzoic acid sample, 25 × 0.46 cm I.D. Zorbax C_8 column, 35% methanol–buffer (25 m*M*, pH 2.9), 30°C, 1 ml/min; (B) aniline sample, conditions as in Table I except pH = 3.00. Solid curves are predicted by DryLab I/mp.

arations from the present study showed similar agreement of experimental and calculated values of *N*. The absence of "non-ideal" effects leading to excessive band broadening for the aniline sample is noteworthy, particularly as no amine modifiers were added to the mobile phase. The StableBond CN column used for these separations seems to be relatively free of these "non-ideal" effects.

Band width anomalies. The above discussion of values of *N* for the benzoic acid and aniline samples has one exception. The compound 3,5-dimethylaniline (DMA) also gave reasonable plate numbers in most separations, but this was not the case for mobile phases with pH values in the range 3.0–4.5. Fig. 3 summarizes the band shape of DMA as a function of pH values that overlap the range 3.5–4.5. For a pH of 3.00, the DMA band tails, but its plate number is normal (*N* = 14 200). For pH values of 3.5–4.0, the band fronts markedly and the plate number is less than 3000. For pH values of 4.5 or higher, the band shape is normal and the plate number is again

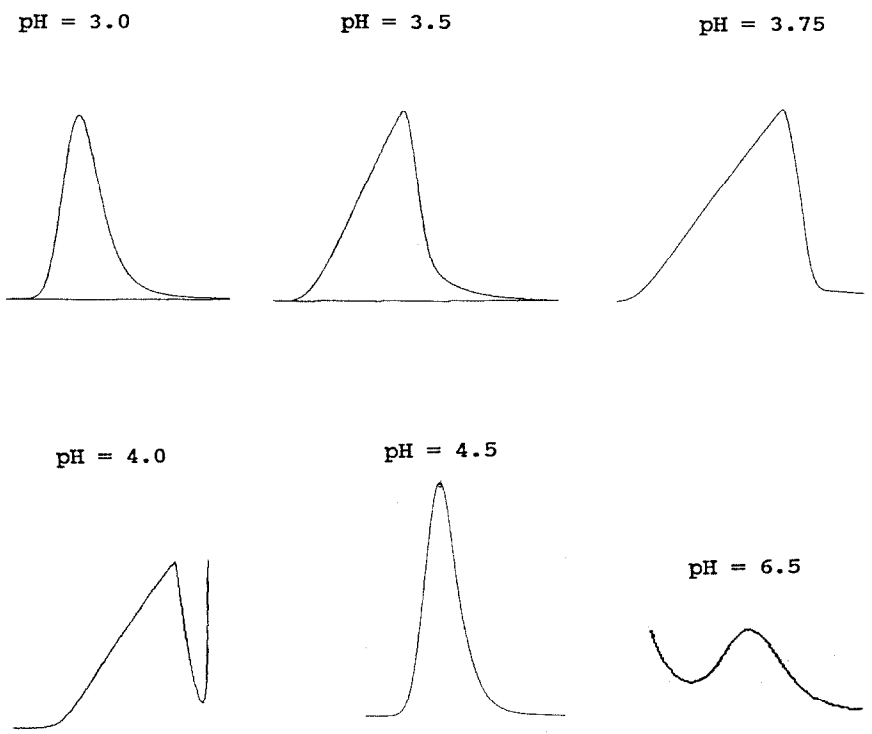

Fig. 3. Anomalous behavior of 3,5-dimethylaniline band. Conditions as in Table I except for indicated pH.

normal (N = 11 400–13 000). The pK_a value of DMA is believed to be about 3.8–4.0 (see Table VII in ref. 1), which approximates the pH of maximum band distortion in Fig. 3. Why this should be so, however, is unclear. A similar example of peak deterioration at intermediate pH values has been reported for the reversed-phase separation of 1-naphthoic acid [19].

The present StableBond CN column is manufactured from a silica that is relatively free from heavy-metal contamination. The excellent band shapes and high plate numbers observed for the remaining substituted anilines (Table I) suggest that this column packing is relatively free of the adverse silanol effects that often result in wider, tailing bands for basic amine solutes. However, the results shown in Fig. 3 emphasize the possibility of unexpected band broadening for some compounds (and some experimental conditions), suggesting a need to take this possibility into account during method development and optimization. Computer simulation (DryLab I/mp or other software) in its present form is unable to predict the behavior shown in Fig. 3. In the example in Fig. 3, this could lead to erroneous predictions of resolution (values that are too high) when the mobile phase pH is between 3.5 and 4.0.

Resolution as a function of pH and %B

Substituted benzoic acid sample. Retention data were reported previously [10] for the separation of the benzoic acid sample as a function of %B and a pH range of 2.6–3.2. These data do not allow a complete optimization of this separation as a function of pH (because of errors in pH extrapolation noted in ref. 1), but a partial optimization is possible. Fig. 4 summarizes our results in the form of resolution maps[a] as a function of pH for different %B values. Fig. 4A–C show resolution maps as a function of pH for values of %B of (A) 35%, (B) 40% and (C) 45%. The resolution (R_s) varies with both pH and %B, and a maximum value of R_s requires optimization of both pH and %B; R_s = 2.0 for pH = 3.08 and 35% B. Fig. 4D shows a similar map for R_s *vs.* %B and pH 2.9; for reasonable values of k', the methanol concentration must be >30%, and the maximum possible resolution is seen to be R_s = 1.6.

Substituted aniline sample. The separation of the aniline sample was carried out for a wide range of pH values (2–6.5) and mobile phases with 25 or 35% B (Table I in ref. 1). This allowed a more complete optimization of pH and %B than was possible for the benzoic acid sample (above). Fig. 5 shows a resolution map *vs.* pH for 25% B over the pH range 2.5–5.0; the resolution at lower and higher pH values is <0.5. The maximum resolution is R_s = 1.4 for pH = 4.1.

We next obtained similar resolution maps (anilines sample) for other values of %B, as in Fig. 4 for the benzoic acid sample. To summarize these data (not shown), no other combination of pH and %B yielded a higher R_s value than could be obtained for 25% B and a pH of 4.1. While unexpected, this result was initially rationalized as being due to the fortuitous initial selection of an optimum %B value (25%). Further study of resolution as a function of %B and pH, however, disclosed a curious finding: there existed a number of pH–%B combinations that gave similar resolution (R_s = 1.0–1.4) as for our optimum pH and 25% B.

We next examined resolution maps of R_s *vs.* %B for different pH values. These revealed an even more surprising finding: maps of R_s *vs.* %B were surprisingly similar to corresponding maps of R_s *vs.* pH, especially when the comparison involves similar values of pH and %B between the two maps. This is illustrated in Fig. 6. Fig. 6A reproduces the resolution map of Fig. 5 for pH = 2.9–3.6. Fig. 6B is an R_s *vs.* %B map for a pH of 3.0, *i.e.*, bracketed by the pH range in Fig. 6A. The two maps are virtually superimposable; not only is the resolution trace the same, but also the critical band pairs (indicated by numbers within the figure, *e.g.*, 10/2 refers to bands 10 and 2) are the same for corresponding regions of each resolution map.

A similar comparison is made in Fig. 6C and D, where the map for R_s *vs.* pH covers the pH range 4.3–4.9, and the R_s *vs.* %B map is for a pH of 4.5. Again, the two maps are remarkably similar. What is the significance of the comparisons shown in Fig. 6? If values of pH and %B are defined for a given separation, *e.g.*, pH = 3.0 and 25% B for the example in Fig. 6A and B, a change in either pH or %B

[a] An average plate number is assumed in these resolution maps, as determined from data for the input runs.

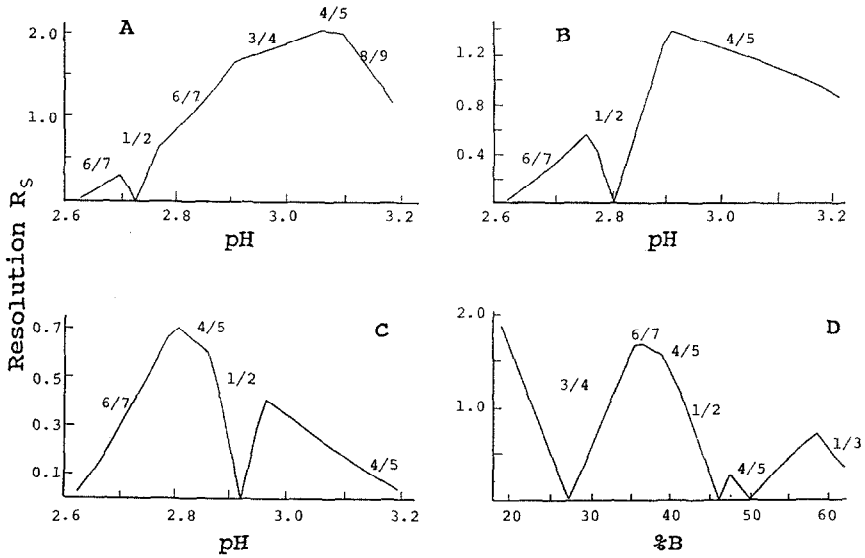

Fig. 4. Resolution maps for the separation of the benzoic acid sample. Conditions as in Fig. 2A except for pH and %B: (A) 35% B; (B) 40% B; (C) 45% B; (D) pH 2.9. Numbers in the figure (*e.g.*, 4/5) refer to "critical" band pairs for which resolution is least.

around these values will produce similar changes in band spacing and resolution. That is, the effects of %B or pH on the separation are not independent but instead highly correlated.

Selectivity as a function of pH and %B. Changes in band spacing or selectivity as a function of pH result from changes in the ionization of acidic or basic sample components. Changes in selectivity as a function of %B reflect differences in the values of

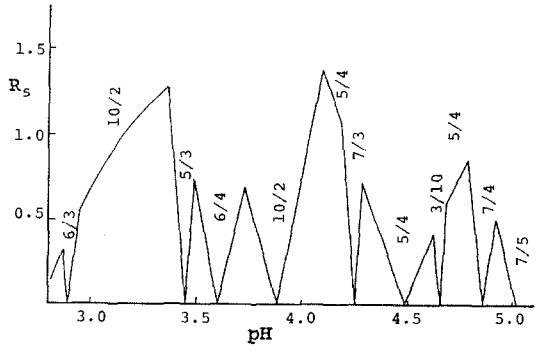

Fig. 5. Resolution map for the separation of the aniline acid sample. Conditions as in Table I, except 25% B and pH varies. Numbers in the figure (*e.g.*, 4/5) refer to "critical" band pairs for which resolution is least.

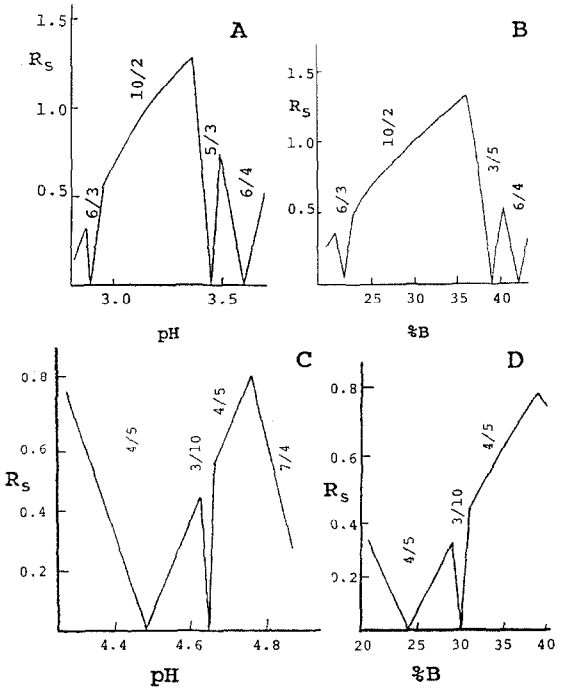

Fig. 6. Resolution maps for the separation of the aniline sample: similarity of changes in selectivity as either pH or %B is varied. Conditions as in Table I, except as noted. (A) 25 %B; (B) pH = 3.0; (C) 25% B; (D) pH = 4.5. Numbers in the figure (*e.g.*, 4/5) refer to "critical" band pair for which resolution is least.

TABLE II

VALUES OF S FOR THE SUBSTITUTED ANILINES AS A FUNCTION OF pH; STABLEBOND CN COLUMN

Derived from the retention data in Table I in Part I [1] by means of eqn. 2. Conditions as in Table I except for pH and %B.

Aniline solute	Parameter	pH									
		2.0	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5
4-Methoxy-	S	–[a]	–	–	–	0.9	1.2	1.6	2.0	2.2	2.3
	F^+	0.95	0.86	0.66	0.38	0.16	0.06	0.02	0.01	0.00	0.00
3-Methyl-	S	–	–	–	0.4	0.9	1.5	1.8	1.9	2.0	2.0
	F^+	0.97	0.92	0.79	0.55	0.28	0.11	0.04	0.01	0.00	0.00
3-Cyano-	S	0.9	1.7	2.0	2.1	2.3	2.4	2.3	2.3	2.2	2.2
	F^+	0.57	0.30	0.12	0.04	0.01	0.00	0.00	0.00	0.00	0.00
2-Chloro-	S	0.9	1.6	1.9	2.0	2.1	2.2	2.1	2.2	2.2	2.2
	F^+	0.55	0.28	0.11	0.04	0.01	0.00	0.00	0.00	0.00	0.00
4-Chloro-	S	–	0.5	0.7	1.2	1.7	2.1	2.2	2.2	2.2	2.2
	F^+	0.95	0.85	0.65	0.37	0.16	0.06	0.02	0.01	0.00	0.00
3-Chloro-	S	0.7	0.7	1.2	1.7	2.0	2.2	2.1	2.2	2.2	2.2
	F^+	0.88	0.70	0.42	0.19	0.07	0.02	0.01	0.00	0.00	0.00
3,5-Dimethyl-	S	–	–	–	0.8	1.8	1.9	2.1	2.4	2.4	2.5
	F^+	0.98	0.94	0.83	0.61	0.33	0.14	0.05	0.02	0.00	0.00
N-Ethyl-	S	–	–	–	0.4	0.7	1.2	1.7	2.1	2.2	2.2
	F^+	0.98	0.94	0.83	0.62	0.34	0.14	0.05	0.02	0.00	0.00
3,4-Dichloro-	S	1.3	2.1	2.6	2.7	2.9	3.1	3.0	3.0	3.0	3.0
	F^+	0.69	0.41	0.18	0.07	0.02	0.01	0.00	0.00	0.00	0.00
3,5-Dichloro	S	2.1	2.6	2.9	2.8	3.0	3.1	3.0	3.0	3.0	3.0
	F^+	0.49	0.24	0.09	0.03	0.01	0.00	0.00	0.00	0.00	0.00

[a] Dashes indicate inaccurate value of S due to small values of k' used in eqn. 2.

S for two adjacent bands in the chromatogram (see eqn. 2 and the discussion in refs. 7 and 9). The results in Fig. 6, which show a marked correlation between changes in selectivity due to variation of either pH or %B thus imply a strong correlation between values of S and the fractional ionization F^+ of these substituted anilines. We therefore examined values of S for these compounds as a function of pH. Table II summarizes these values of S and includes corresponding values of F^+ calculated via eqns. 3–5 in Part I [1], using values of K_a from Table II in Part I [1] for $pH_2 = 3.0$.

For pH > 5.0, the various components of the aniline sample are almost completely non-ionized: $F^+ < 0.05$ (Table II). Over this range of pH it is seen in Table II that values of S are independent of pH, as is normally the case for neutral solutes. For lower pH values, however, where $F^+ > 0.05$, the values of S are seen to decrease sharply. This is further shown in Fig. 7, where the ratio of S to its value at high pH (S°) is plotted *vs.* fractional ionization F^+; values of S/S° (open circles) are seen to decrease regularly as F^+ increases[a]. We shall show in a following section that the dependence of S on F^+ in Fig. 7 is consistent with the similarity of band spacing changes that results from changes in either pH or %B (Fig. 6).

Also shown in Fig. 7 (dashed line) is the dependence of S on solute ionization (F^-) for the benzoic acid sample. While there is a tendency toward lower

[a] The closed circles in Fig. 7 are derived from k' values that are < 1 and therefore less accurate; we can ignore these data points for the purpose of discussion.

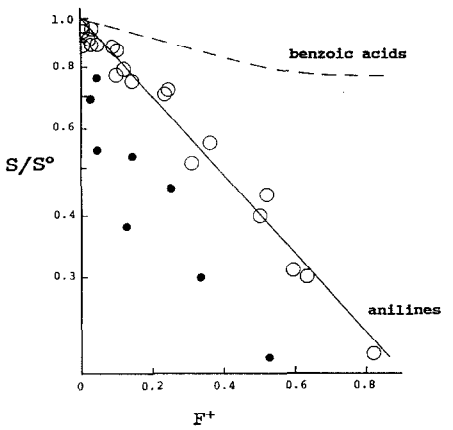

Fig. 7. Dependence of values of S (eqn. 2) on fractional ionization, F^+, of solute. Data in Table II for anilines (solid line); dashed line, data for benzoic acids (conditions as in Fig. 1; see ref. 9 for raw data). ● = Data which are considered to be of low accuracy owing to small values of k' (see text); ○ = other data.

values of S for more highly ionized solutes in this sample, the dependence of S on F^+ is *much* less pronounced. This smaller change in S with solute ionization is also consistent with our finding that band spacing changes that result from a change in pH or %B are *not* the same for the benzoic acid sample. That is, the simultaneous variation in pH and %B as a means of maximizing sample resolution is much more effective for the benzoic acid sample than for the aniline sample.

Simple description of the basis of the similar dependence of selectivity on pH and %B for the aniline sample. The chromatograms in Fig. 1 show that eight of the ten components of the aniline sample elute early, with the last two bands (3,4- and 3,5-dichloroaniline) eluting much later (see also Table I in Part I [1]). Sample resolution is determined mainly by overlapping bands within the first group of eight compounds (excluding the dichloroanilines). It can be seen in Table II that the $S°$ values of these early-eluting anilines are relatively constant, *i.e.*, the value of S for pH > 5.0 ranges from 2.0 to 2.5. Assume for the moment that the $S°$ values for this group of compounds are about the same.

Next assume that two of these bands overlap completely for some value of pH. This situation is illustrated in the hypothetical example in Fig. 8A, where k' is plotted *vs.* pH for two bands X (solid

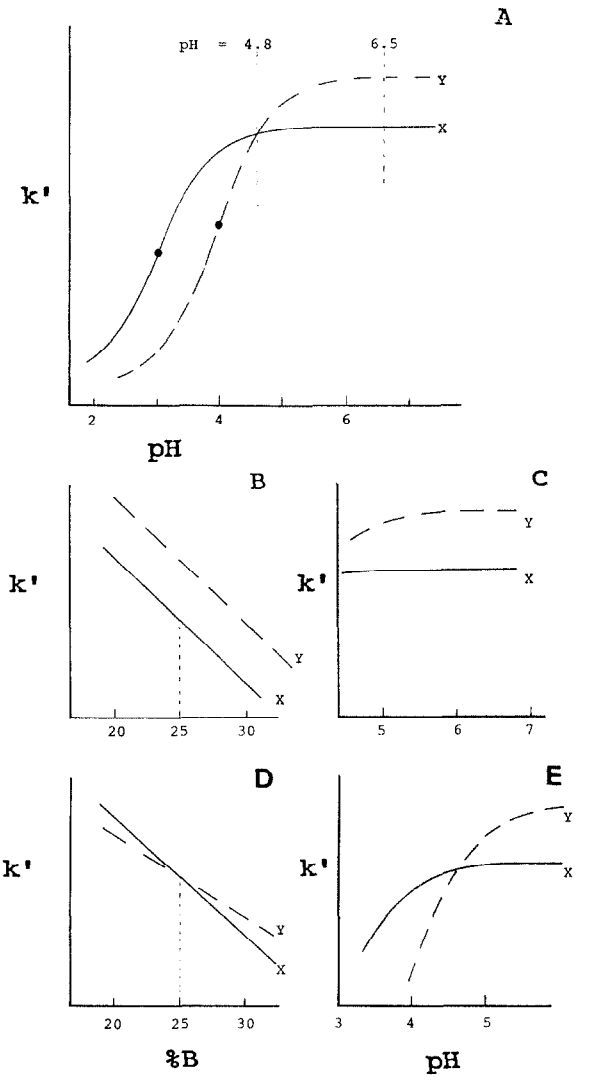

Fig. 8. Hypothetical example that further illustrates the basis of similar changes in band spacing as a function of either pH or %B for the aniline sample, assuming 25% B; see text for further details. (A) Plots of k' *vs.* pH for solutes X and Y; pK_a = 3 for X and 4 for Y (●); (B) plot of k' *vs.* %B for pH = 6.5 (see A); (C) plot of k' *vs.* pH for 25% B; (D) plot of k' *vs.* %B for pH = 4.8 (see A); (E) plot of k' *vs.* pH for 25% B.

curve) and Y (dashed curve); we assume also that %B = 25%. Band X has a pK_a value of 3.0 and the pK_a value for band Y is 4.0 (solid circles in Fig. 8A). The two bands overlap completely for pH = 4.8. Now consider the separation of the two bands as experimental conditions (pH and %B) are varied

TABLE III

RETENTION TIMES (min) FOR SUBSTITUTED ANILINE SAMPLE AS A FUNCTION OF pH AND %B

Conditions: 25 × 0.46 cm I.D. StableBond C_8 column, other conditions as in Table I.

Methanol concentration (%)	Compound	pH					
		2.5	3	3.5	4	4.5	5
30	*p*-Anisidine	3.18	3.46	3.59	4.62	5.44	6.63
	3-Aminobenzonitrile	9.04	9.83	10.29	10.48	10.48	10.56
	m-Toluidine	4.18	4.88	6.23	9.77	12.18	13.88
	4-Chloroaniline	6.87	10.09	14.09	18.45	19.32	19.91
	3-Chloroaniline	10.72	14.90	18.27	20.60	20.88	21.13
	2-Chloroaniline	17.71	19.54	20.39	20.86	20.84	20.74
	N-Ethylaniline	3.84	4.54	6.32	11.80	18.69	25.74
	Nitrobenzene	24.83	24.79	24.77	24.86	24.69	24.84
	3,5-Dimethylaniline	6.79	7.91	10.69	19.32	23.74	28.32
	3,4-Dichloroaniline	41.84	49.42	54.75	55.29	54.65	55.29
	3,5-Dichloroaniline	75.91	80.70	83.58	82.69	80.75	81.44
40	*p*-Anisidine	3.01	3.21	3.35	4.06	4.40	5.13
	3-Aminobenzonitrile	6.45	6.64	6.7	6.69	6.65	6.68
	m-Toluidine	3.73	4.51	5.52	7.46	8.31	9.02
	4-Chloroaniline	6.61	8.72	10.27	11.25	11.52	11.64
	3-Chloroaniline	8.99	10.82	11.79	12.13	12.31	12.19
	2-Chloroaniline	11.74	12.09	12.41	12.47	12.43	12.32
	N-Ethylaniline	3.50	4.35	5.7	9.82	12.90	15.89
	Nitrobenzene	14.57	14.35	14.58	14.37	14.43	14.37
	3,5-Dimethylaniline	5.08	6.28	8.32	12.28	14.06	15.64
	3,4-Dichloroaniline	23.27	24.42	25.94	25.55	26.16	25.50
	3,5-Dichloroaniline	36.55	36.65	38.10	37.00	37.98	36.85

for an initial separation with a mobile phase of 25% B and pH = 6.5. Fig. 8B and C show the variation of k' as a function of %B (pH = 6.5) and pH (25% B). Because the two compounds are essentially non-ionized in this pH range, the values of $S = S^\circ$ and are therefore the same (we assumed equal S° values). Fig. 8B shows that band spacing does not change as %B is varied[a], because the S values for each band are the same. Similarly, band spacing does not change as pH is varied (Fig. 8C), because the ionization of the two compounds remains unchanged for small changes in pH.

Now consider how separation changes with pH and %B in the region of pH = 4.8, where the two bands overlap (Fig. 8A). Because band B is significantly ionized for pH = 4.8, its value of S will decrease significantly (Fig. 7). Band X is not significantly ionized at pH = 4.8, so its value of S remains about the same as at pH 6.5. Fig. 8D reflects this situation, where the slope of the k' *vs.* %B plot for band X is seen to be greater than that for band Y. As a result, an increase in %B leads to the later elution of band Y *vs.* band X, and the separation of the two bands for %B values either greater or less than the original values of 25%.

Fig. 8E shows a similar situation as pH is varied; band Y is more retained for higher pH values than band X, and the two bands can be separated by either an increase or decrease in pH. Thus for these two bands, our preceding discussion suggests that an increase in %B will give a comparable change in band spacing and resolution as for some increase in pH.

The examples in Fig. 8 can be repeated for every pair of bands in the sample. The result will then be similar resolution maps for the sample as a function

[a] Straight-line plots as in Fig. 8B and D are expected when log k' is plotted *vs.* %B (eqn. 2). Here we have ignored the actual curvature of plots of k' *vs.* %B.

TABLE IV

VALUES OF S FOR THE SUBSTITUTED ANILINES AS A FUNCTION OF pH; STABLEBOND C_8 COLUMN

Derived from the retention data in Table III by means of eqn. 2. Conditions as in Table III.

Aniline solute	Parameter[a]	pH					
		2.5	3.0	3.5	4.0	4.5	5.0
4-Methoxy-	S	–[b]	–	1.2	1.4	1.9	2.0
	F^+	0.86	0.66	0.38	0.16	0.06	0.02
3-Methyl-	S	–	0.8	0.97	1.7	2.2	2.4
	F^+	0.92	0.79	0.55	0.28	0.11	0.04
3-Cyano-	S	2.2	2.5	2.7	2.8	2.9	2.9
	F^+	0.30	0.12	0.04	0.01	0.00	0.00
2-Chloro-	S	2.2	2.5	2.6	2.7	2.7	2.7
	F^+	0.28	0.11	0.04	0.01	0.00	0.00
4-Chloro-	S	0.3	0.9	1.8	2.6	2.7	2.8
	F^+	0.85	0.65	0.37	0.16	0.06	0.02
3-Chloro-	S	1.0	1.7	2.3	2.7	2.7	2.8
	F^+	0.70	0.42	0.19	0.07	0.02	0.01
3,5-Dimethyl-	S	–	–	1.5	2.4	2.7	2.9
	F^+	0.94	0.83	0.61	0.33	0.14	0.05
N-Ethyl-	S	–	0.4	0.7	1.1	1.9	2.4
	F^+	0.94	0.83	0.62	0.34	0.14	0.05
3,4-Dichloro-	S	2.8	3.3	3.5	3.6	3.4	3.6
	F^+	0.41	0.18	0.07	0.02	0.01	0.00
3,5-Dichloro	S	3.3	3.6	3.6	3.7	3.4	3.6
	F^+	0.24	0.09	0.03	0.01	0.00	0.00

[a] Values of F^+ are the same as for Table II (not corrected for difference in %B).
[b] See footnote in Table II.

of either %B or pH, as seen in the experimental examples in Fig. 6. A more fundamental question, however, is *why* the values of S for the anilines correlate with the fractional ionization of these solutes. Also, why do the benzoic acids not show a similar behavior? We shall not address these questions here, but they are surely worthy of further investigation.

C_8 vs. cyano columns. One experimental difference between the separations reported here of the anilines and benzoic acids is the use of a cyano column for the aniline separations and a C_8 column for the benzoic acids. It might be argued that silanol effects preferentially affect the retention of anilines *vs.* benzoic acids, and a cyano column is likely to have a greater number of accessible silanols. For these reasons we repeated our study of aniline retention as a function of pH, but substituted a C_8 column for the original cyano column.

Table III summarizes retention times as a function of pH for a C_8 column and two different %B values, 30% and 40%. Table IV provides derived values of S for each solute as a function of pH (Table III and eqn. 2). A similar pattern of decrease in S with increase in solute ionization (value of F^+) is observed as in Table II for the cyano column. We have not yet attempted a more detailed comparison of the data in Tables II and IV.

Optimizing separation as a function of %B and pH

When changes in resolution as a function of mobile phase pH and %B are not equivalent, as in the case of the benzoic acid sample, it is obviously useful to be able to map separation as a function of all

TABLE V

SUMMARY OF MAXIMUM RESOLUTION AND RELATED k'-RANGE AS A FUNCTION OF BOTH pH AND %B FOR THE SUBSTITUTED ANILINES SAMPLE

25% B, pH varied			
pH		R_s	k' range
3.35		1.3[a]	0.4–16
4.10		1.4[b]	0.6–16
pH 3–4.5, %B varied			
pH	%B	R_s	k' range
2.5	42	1.0[a]	0.1–5.8
3.0	36	1.3[a]	0.2–7
3.5	21	1.3[a]	0.5–20
	36	1.1[b]	0.2–8
4.0	28	1.0[a]	0.5–13
	38	0.6[b]	0.4–7
4.5	39	0.8[b]	0.6–5.8

[a] Bands 10/2 critical.
[b] Bands 5/4 critical.

possible values of pH and %B. In this way the best compromise can be obtained in terms of resolution, run time and k' range. When the effects of pH and %B on band spacing are similar, as for the case of the anilines, the usefulness of this same approach is more limited. Table V summarizes the results of combined %B *vs.* pH mapping for the anilines.

Two primary resolution maxima exist in the map of Fig. 5 for 25% B, at pH 3.4 and 4.1. These resolution maxima are defined by two different overlapping bands: 10/2 and 5/4[a]. Table V shows that as pH is increased, the 10/2 maximum[a] shifts to lower values of %B, but the maximum resolution when this band pair is limiting remains relatively constant ($1.0 < R_s < 1.3$). The 5/4 maximum[a] occurs at about the same value of %B as pH is varied, and the maximum resolution for this band pair occurs for about pH 3.5.

A more interesting observation in Table V is that for roughly the same maximum resolution, the mobile phase composition (%B and pH) can be varied to adjust the k' range of the sample. For $R_s > 1.0$, the k' range can be varied from 0.1–5.8 to 0.6–16. As $k' < 0.5$ should be avoided, this favors the choice of 25% B and a pH = 4.1 in this example.

[a] In Fig. 2 bands 2 and 10 are 3-methyl- and N-ethylanilines; bands 4 and 5 are 2- and 4-chloroanilines.

CONCLUSIONS

Column efficiency was studied for the separation of the present samples composed of substituted anilines and benzoic acids as a function of mobile phase pH. Comparisons of experimental band widths with values predicted by computer simulation showed reasonable agreement: ±6% for the benzoic acids and ±11% for the substituted anilines. With the exception of 3,5-dimethylaniline (DMA), plate numbers for the aniline sample did not vary with pH over the range 2–6.5. DMA showed anomalous band-broadening behavior in the pH range 3.5–4.0; strong fronting and excessive band broadening were seen. For pH < 3.5 and > 4.0, band broadening for DMA was similar to that for the other anilines in this sample.

The use of the simultaneous variation of mobile phase pH and %B values was studied for both the benzoic acid and aniline samples. With the benzoic acids, it was useful to vary each of these two variables for maximum sample resolution. The aniline sample behaved differently in this respect. It was found that changes in selectivity as a function of either pH or %B were similar, and either variable could be used to achieve a similar maximum resolution for the aniline sample.

Further study showed that the slope of the retention *vs.* %B plot (value of S) for each aniline was strongly dependent on sample ionization; values of S decrease markedly when the extent of ionization of an aniline increases. This observation explains the similar band spacing changes that result when either pH or %B is varied for the aniline sample. A similar decrease in values of S as the sample ionization increases was noted for the benzoic acid sample, but the magnitude of this effect was much smaller. No explanation for the fundamental cause of these observations has been found.

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