CHROMSYMP. 2029

High-performance liquid chromatographic computer simulation based on a restricted multi-parameter approach

I. Theory and verification

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ABSTRACT

A computer program (DryLab MP®) is described for the simulation of HPLC separations where two or more variables (*e.g.*, temperature and pH) are changed simultaneously. It is assumed that preliminary method development has resulted in a mobile phase of appropriate strength, such that $1 < k' < 20$ for all bands in the chromatogram. If it is desired to simulate separation as a function of changes in the values of n variables, then one or two additional runs are carried out for each variable —changing only that variable. Retention times for each of the latter runs are entered into the computer, and predictions of separation as a function of all conditions are now possible. Because simultaneous changes in two or more variables can lead to significant interaction effects and less accurate predictions, the software evaluates each simulation for possible errors. Allowed conditions must be capable of predicting values of α with an accuracy better than $\pm$ 2% (1 S.D.).The present computer program has a number of possible applications during and following method development, as discussed in the following paper (Part II).

INTRODUCTION

Users of high-performance liquid chromatography (HPLC) are faced with various problems during method development and the routine application of a final procedure. For samples of any complexity, the selection of experimental conditions that can provide an adequate separation can be a considerable challenge [1]. Once a suitable method is developed, other laboratories may experience difficulty in obtaining the same separation; *i.e.*, the method is not sufficiently rugged. When a replacement column is required, it may be found that columns from different batches do not yield the same separation [2] —requiring modification of the original procedure. Finally, other problems can arise as a result of errors in formulating the mobile phase (including errors in the measurement of pH), setting the proper flow-rate, etc. [2].

Procedures for HPLC method development vary widely among different laboratories. Many chromatographers adopt a trial-and-error approach, although this is often inefficient. Several method development schemes have been described which utilize a defined number of experimental runs to predict (and optimize) separation for

a wide range of conditions [1,3–5]. These latter procedures can be divided into two groups, depending on whether one or several separation parameters are varied. When only one parameter is varied, two or three initial experimental runs usually suffice for computer-assisted mapping of retention and separation. When two or more separation variables are to be mapped, the number of initial experimental runs increases rapidly. Multi-variable mapping is more likely to uncover a near-optimum separation, but the amount of work required can be prohibitive.

In this paper we describe a somewhat different approach to HPLC method development, based on "restricted" multi-parameter mapping of retention and resolution. This procedure can be used from the beginning of method development, or it can complement procedures based on the optimization of a smaller number of experimental parameters. The software we will describe for multi-parameter mapping can also be applied to the various problems noted above: lack of method ruggedness, variations in retention from column to column, etc.

THEORY

The dependence of HPLC retention (values of k') on different separation variables has been studied rather thoroughly, and various equations have been reported which describe these results. When only one separation parameter is varied at a time, these theoretical or empirical relationships are often of rather simple form and are reasonably accurate. They are thus ideally suited to single-parameter mapping of separation —including both interpolation and extrapolation of initial experimental data. When two or more parameters are varied simultaneously, the resulting equations are usually much more complex —and also less reliable. As a result, multi-parameter mapping usually employs general-purpose (empirical) fitting equations which require a larger number of data points (initial runs).

In the present treatment we have tried to combine the best features of single- and multi-parameter mapping, by determining under what conditions the simple relationships for the single-parameter case can be extended to multi-parameter mapping. In the limiting case where the variation of all parameters is sufficiently restricted, it can be assumed that there will be no interaction among the different variables, so that retention for a change in one or more variables i is given as

$$k' = k_R \prod^{i} (k_i/k_R) \tag{1}$$

Here k_R refers to a value of k' for a given band in the reference (starting) run, and k_i is the value of k' for a run where only variable i is changed. Thus the dependence of k' on i is determined with other variables unchanged (same values as in reference run), so that k_i/k_R can be calculated for any value of each variable i.

For small enough variations in the different variables i, eqn. 1 can be written in an equivalent form

$$k' = k_R + \sum^{i} (k_i - k_R) \tag{2}$$

However the following treatment is based on eqn. 1 rather than eqn. 2.

TABLE I

VARIABLES THAT AFFECT RETENTION IN REVERSED-PHASE AND ION-PAIR CHROMA-TOGRAPHY

Group	Non-ionizable solutes	Ionizable solutes
I[a]	Organic solvent (mixtures of methanol, acetonitrile, THF)	pH, ion-pair-reagent concentration
II	%B, column type	%B, temperature, column type, additive[b] concentration
III	Temperature	Ion-pair-reagent type

^a Variables from group I are most likely to be useful for controlling values of α and band spacing; group III variables are least promising [1].
^b Buffer, amines, salt, etc.

Retention relationships (single parameter)

Those experimental conditions which have been used to control retention in HPLC are summarized in Table I (see discussion in ref. 1, Ch. 4 and 5). They are grouped (I–III) according to their relative potential for use in controlling band spacing (values of α). While changes in the parameters listed in group I of Table I are more likely to create large changes in α, it is our premise that any or all of the remaining variables can be of potential value for a given sample —especially samples which prove more difficult to separate. The present treatment will emphasize changes in mobile phase composition and temperature.

Solvent strength (%B). Numerous studies [6,7] have established that retention in reversed-phase and ion-pair chromatography is usually well approximated by the empirical relationship

$$\log k' = \log k_w - S \varphi \tag{3}$$

Here k' is the capacity factor of the solute in a mobile phase A/B, φ is the volume fraction of the organic solvent B, k_w is the (extrapolated) value of k' for water (solvent A) as mobile phase, and S is a constant whose value depends on the solute and (to a lesser extent) experimental conditions.

The value of α for two solutes i and j is then given by

$$\log \alpha = \log (k_{wj}/k_{wi}) + (S_i - S_j) \varphi$$

$$= (\text{constant}) + \Delta S \varphi \tag{4}$$

Here subscripts i and j refer to the respective solutes. The value of ΔS for two solutes thus determines the effect of a change in φ (or %B) on band spacing and α.

Small deviations of experimental data from eqn. 3 are often observed, but these usually have little effect on the accuracy of this relationship over a range in values of φ corresponding to $1 < k' < 20$ for all sample components [8].

Temperature (T). In chromatography (as for other chemical or physical

processes) the enthalpy and entropy of retention can usually be assumed constant over some range in temperature, leading to a dependence of retention on temperature of the form

$$\log k' = A + \bar{B}/T \tag{5}$$

where A and $\bar{B}$ are constants for a given system, and T is the absolute temperature (K). Numerous examples of the applicability of eqn. 5 for HPLC systems have been reported [9–11].

The value of α for two solutes i and j is given by

$$\log \alpha = (A_j - A_i) + (\bar{B}_j - \bar{B}_i)\,(1/T)$$
$$= (\text{constant}) + \Delta\bar{B}\,(1/T) \tag{6}$$

The value of $\Delta\bar{B}$ for the two solutes thus determines the effect of a change in temperature on band spacing and α.

pH. The effect of pH on sample retention has been described in detail by several workers [12–16]. The ionization of the solute is defined by its K_a value, and the observed k' value is equal to the sum of k' values for the ionized and non-ionized forms of the solute:

$$k' = k^\circ\,(1 - F^\pm) + k^\pm\,F^\pm \tag{7a}$$

Here $k^\pm$ and k° refer to the values of k' for the solute in the ionized and non-ionized forms, respectively; $F^\pm$ is the fraction of solute molecules that are ionized; *i.e.*,

acids $$F^- = 1/\{1 + ([H^+]/K_a)\} \tag{7b}$$

bases $$F^+ = 1/\{1 + (K_a/[H^+])\} \tag{7c}$$

Eqns. 7a–c have been shown to provide accurate predictions of the retention of acids and bases as a function of pH.

Values of K_a, $k^\pm$ and k° can, in principle, be derived from measurements of k' at three values of pH. In practice, this is complicated by small errors in the measurements of (a) the mobile phase pH and (b) individual values of k'. Optimally, the mobile phase pH values will bracket the pK_a value of the solute and be spaced over a 1–2 unit pH range.

Concentrations of buffer, ion-pair reagent, salt, amine modifiers, etc. The effect on sample retention of these mobile phase additives is usually complex, as discussed by several authors [13,14,16]. Over a small range in concentrations, retention can often be described [17,18] by an equation of the form

$$\log k' \approx C + D \log [X] \tag{8}$$

Here, C and D are constants for a given solute and HPLC system, and [X] refers to the concentration of the mobile phase additive. The approximate nature of eqn. 8 must be

stressed; it is unsuitable for extrapolation beyond the range of values of [X] used to determine C and D.

Mixtures of organic solvents. Retention is often mapped for ternary-solvent mobile phases during method development [1,4,5]. In some cases, the concentration ratio of two organic solvents is varied while holding %B constant (B refers to the sum of the two solvents). In other cases, two binary solvents A/B and A/C having different values of φ are blended. In either case, the resulting dependence of k' on mobile phase composition is complex, and no theoretical equations have been demonstrated to be generally reliable. The use of quadratic equations with interaction terms appears to give a reasonable fit in most cases [19].

Retention relationships (multi-parameter)

These are necessarily based on some model of solute retention, and there is still a general lack of agreement on these retention models for different systems (*cf.*, *e.g.*, the discussion of Horváth in ref. 16 concerning reversed-phase ion-pair systems). Even where physically reasonable models can be derived, the many fitting parameters require a corresponding (large) number of initial experiments, and the reliability of these models over a significant range in values of different variables is often poor. This is particularly true when the objective is the accurate mapping of resolution as a function of separation conditions, since then values of α must be accurate within (at most) a few percent.

Interaction effects. The effects of %B, T, pH and other variables X on the retention of a given solute are given by the parameters S, $\bar{B}$, (K_a, k°, k^+), and D, respectively (eqns. 3, 5, 7a–c). By "interaction effects" we refer to the dependence of these solute parameters on other variables; *e.g.*,

$$S = f(T, \text{pH}, [X_1], [X_2], \ldots) \tag{9a}$$

$$\bar{B} = f(\%B, \text{pH}, [X_1], [X_2], \ldots) \tag{9b}$$

$$D_1 = f(\%B, T, \text{pH}, [X_2], \ldots) \tag{9c}$$

$$D_2 = f(\%B, T, \text{pH} [X_1], \ldots) \tag{9d}$$

$$K_a = f(\%B, T, [X_1], [X_2], \ldots) \tag{9e}$$

$[X_1]$, $[X_2]$, ... refer to the concentrations of different mobile phase additives.

Since the dependence of retention on pH involves three separate parameters (K_a, k°, $k^\pm$), it will prove convenient to ignore changes in these parameters with %B, T, X, etc. by means of the following convention. In every case we begin with a reference set of experimental conditions and a corresponding chromatogram. The problem is then to predict how retention will change as these conditions are varied. If pH is one of the variables, it will be assumed that a change in pH is carried out *first*, so that the effect of pH on retention can be described in terms of the values of K_a, k°, $k^\pm$ for the reference run. Subsequent changes in retention as a result of a change in other parameters (%B, T, X, ...) can then be described in terms of eqn. 9a–e.

Consider next the case where two variables are changed simultaneously; *e.g.*,

%B and temperature. Eqn. 9a–e provide a generalized description of the resulting changes in retention. Thus, a change in %B and T can be described as a two-step process, involving a change in T (for which the reference value of $\bar{B}$ applies), followed by change in the %B (note eqn. 1). It is assumed that values of S and $\bar{B}$ for the reference run have been determined by one-at-a-time changes in %B and T. Retention for the new values of %B and T is thus determinable from the reference value of $\bar{B}$ and a value of S at the new temperature T (S_T). The question is then: how much does S vary with temperature (eqn. 9a)? Alternatively, if S does not vary with temperature, it means that eqn. 1 is applicable for the prediction of retention at the new values of %B and T (i.e., interaction effects are negligible).

Example of variation of S with temperature. A detailed study of retention as a function of temperature and %B has been published for some (predominantly) non-ionic compounds [20]. We will use these data to illustrate our approach to determining the error in predictions based on eqn. 1 (ignoring interaction effects). Values of S for nine solutes at different temperatures can be determined from the data in ref. 19, as summarized in Table II; values of S are shown for 30°C, and the change in S (δS) is shown between 30°C and other temperatures. If values of δS were zero for all solutes and temperatures, this would correspond to no error in the application of eqns. 1, 3 or 5 for changes in %B of up to 20% and changes in temperature of up to 30°C.

The actual values of δS are seen to vary systematically with temperature, reflecting the significance of eqn. 9a. If all solutes exhibited the same value of δS at a given temperature, this would mean that all values of k' are changed by the same ratio (eqn. 3). Consequently there would be no change in values of α, and no error in

TABLE II

VARIATION OF S WITH TEMPERATURE

Conditions: 15 × 0.46 cm I.D. C_8 column; 50 and 70% methanol–water mobile phases used to calculate values of S (eqn. 3); 1 ml/min flow-rate; temperatures as indicated; data calculated from ref. 19.

Solute	S	δS		
	30°C	41–30°C	51–30°C	59.5–30°C
p-Nitrophenol	2.73	$(+0.04)^a$	−0.37	−0.47
Phenol	2.57	−0.27	−0.34	−0.48
Acetophenone	2.93	−0.27	−0.42	−0.55
Methyl benzoate	3.28	−0.21	−0.43	−0.45
Anisole	2.97	−0.23	−0.37	$(-0.19)^a$
Benzene	2.88	−0.25	−0.33	−0.45
Phenetole	3.39	−0.21	−0.36	−0.42
Toluene	3.35	−0.26	−0.37	−0.37
Ethylbenzene	3.80	−0.21	−0.34	−0.50
Av. δS^b		−0.24 ± 0.02	−0.37 ± 0.03	−0.46 ± 0.06
Error in k'		−12 ± 1%	−19 ± 1%	−24 ± 3%
Error in α		± 1.4%	± 2.0%	± 4.0%

[a] Out-of-line value, not included in calculations of error.
[b] Average values of δS for a given temperature change (e.g., 30 to 41°C) with S.D. values (see discussion of text and Fig. 1); the errors in k' and α correspond to average errors in these quantities as predicted from eqn. 1 and 30°C as reference temperature.

predicted values of α. The latter hypothesis comes close to approximating the data of Table II, as seen in the average values of δS listed for each temperature (deviations from this value expressed as 1 S.D.).

Thus for a change in %B from 50 to 70% the average decrease in S by 0.24 units at 41 *vs.* 30°C (Table II) corresponds to an average change in log k' of (change in S) (change in φ) = $(-0.24) \times (-0.2) = -0.048$; *i.e.*, eqn. 3. This is equivalent to a 12% increase in the actual value of k' at 41°C and 70% B. This increase in k' due to the interaction of temperature and %B (eqn. 9a) is relative to the value of k' calculated from eqn. 1, so that the resulting error in eqn. 1 is -12% for this case.

The corresponding error in values of α determined from eqn. 1 is given by the deviation of values of S from the average; *e.g.*, ± 0.02 units at 41 *vs.* 30°C (see Table II). That is, values of S at 41°C are not all exactly 0.24 units lower than values at 30°C; otherwise, there would be no error in values of α from eqn. 1. The average error of ± 0.02 units in S for each solute leads to an error in the difference of S-values (eqn. 4) of $2^{0.5} \times 0.02 = 0.03$ units, which, multiplied by 0.2 (eqn. 4, change in %B from 70 to 50%), leads to an error in predicted values of $\alpha = 1.4\%^{a}$.

If our goal is to maintain the accuracy of calculations of α by eqn. 1 better than ± 2%, it is seen that the system in Table II can be varied by 20% B and 21°C^{b}. There is no reason to believe that other HPLC separations which involve non-ionized solutes will be much different in this respect, suggesting that eqn. 1 should be valid (for predictions of α) over a rather wide range in %B and T. Because mixtures of ionizable acids and bases involve an additional temperature-dependent process (the change of K_a with T), we can anticipate that eqn. 1 will be less reliable for such samples. This will prove to be the case.

The prediction of values of k' by means of eqn. 1 is illustrated for the data of Table II in Table III (DryLab MP calculations). Here the reference conditions are selected as 50% B and 30°C, and values of S and B were determined from runs at 60% B and 41.5°C, respectively. Because the change in %B is only 10% in Table III *vs.* 20% in Table II, the errors in k' and α are predicted to be half as great in the comparisons of Table III *vs.* those of Table II for 41°C (-12% in retention, ± 1.4% in α); *i.e.* predicted errors of -6% in k' and ± 0.7% in α^{c}.

EXPERIMENTAL

Equipment and materials

Separations reported here were carried out on a Beckman/Altex System Gold HPLC system (Beckman, San Ramon, CA, U.S.A.). A 25 × 0.46 cm I.D. Zorbax™ C$_8$ column was used with various methanol–water mobile phases, buffered with acetic

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^{a} These estimated errors in values of α from eqn. 1 are actually *maximum* values, since they include random errors in the measurement of the values of S in Table II.

^{b} The prediction of retention times from eqn. 1 will be in error by -24%, however (Table II).

^{c} The larger error in α in Table III (± 1.7% *vs.* 0.7% predicted from Table II) reflects a greater contribution of experimental error in these calculated values of α. That is, the errors in α of Table II are due primarily to changes in S with T, whereas the α values of Table III reflect additional random experimental error in the measurements of the three experimental k' values used to calculate α (k' values for 50% B/30°C, 50% B/41.5°C and 60% B/30°C runs), as well as error in the measurement of k' values for the 60% B/41.5°C run.

TABLE III

PREDICTIONS OF RETENTION (EQN. 1) FOR SYSTEM IN TABLE II

$T = 41.5°C$, 60% methanol.

Solute	k'		α	
	expt.	calc.	expt.	calc.
p-Nitrophenol	0.42	0.42	1.00	$(0.94)^a$
Phenol	0.42	0.40	1.64	1.63
Acetophenone	0.69	0.65	1.80	1.84
Methylbenzoate	1.24	1.20	0.99	0.96
Anisole	1.23	1.15	1.08	1.11
Benzene	1.33	1.28	1.50	1.52
Phenetole	2.00	1.93	1.16	1.15
Toluene	2.31	2.22	1.66	1.66
Ethylbenzene	3.84	3.69		
Error	$-4\pm3\%$		$\pm1.7\%$	

^{a} Out-of-line value, not included in error calculation.

acid–sodium acetate (column dead-time $t_0 = 2.56$ min). Solvents and reagents were of HPLC grade. Water was purified with a Milli-Q system (Millipore, Milford, MA, U.S.A.). The various solutes (see Table IV) were obtained from Aldrich (Milwaukee, WI, U.S.A.).

Software
    Computer simulations were carried out by means of DryLab MP software (LC Resources, Lafayette, CA, U.S.A.), using an IBM-compatible personal computer (IBM-AT) with a math-coprocessor.

RESULTS AND DISCUSSION

    The present approach to multi-parameter mapping of resolution for application to HPLC method development (and related problems in the routine laboratory) is illustrated in Fig. 1. An initial gradient elution separation (Fig. 1A) is carried out in order to determine an approximately optimum %B for an isocratic separation (see discussion in pp. 239–244 of ref. 1). This latter run (Fig. 1B) then becomes the reference run for further method development (Fig. 1C) via multi-parameter computer simulation. The example of Fig. 1 assumes that %B, temperature and pH will be varied. Therefore additional runs as indicated in Fig. 1C are carried out (dark circles, changing %B by 10%, T by 10°C and pH by ±0.6 units).
    As a result of interaction effects, it is not possible to vary two or more separation conditions simultaneously for every value of these parameters —and achieve accurate predictions. This is illustrated schematically in Fig. 2, which indicates the various combinations of two variables which are allowed; *i.e.*, which provide an accuracy in predictions of α of better than $\pm2\%$ (1 S.D.). The simultaneous variation of all three of these parameters is also possible, within limits that will shortly be defined. The example
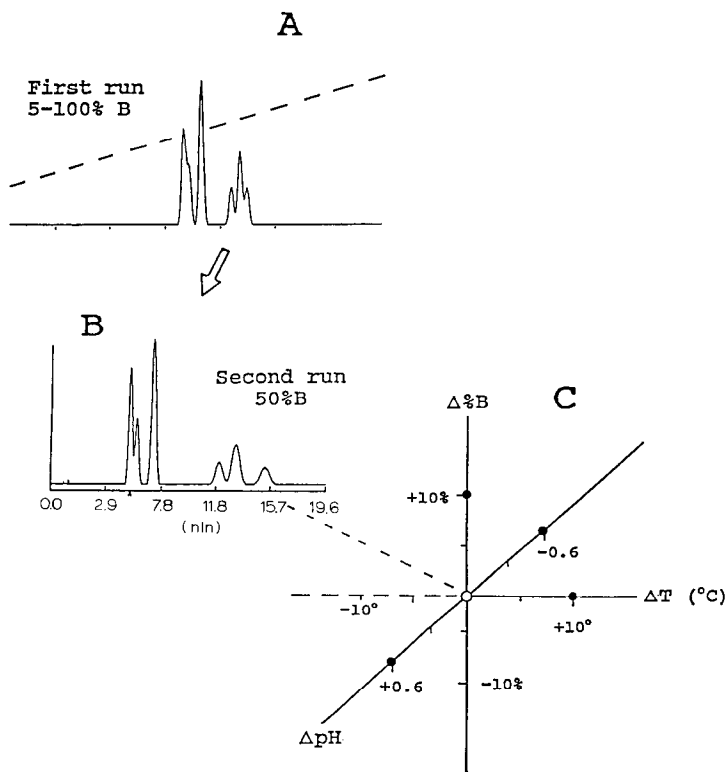
Fig. 1. Hypothetical representation of simplified multi-parameter mapping (interaction effects ignored). (A) Initial gradient elution run; (B) isocratic run predicted from run A; (C) multi-parameter mapping using run B (open circle at center of diagram) as reference run. Closed circles correspond to runs 3–6 (change in %B, T or pH vs. reference run).

of Figs. 1 and 2 can be extended to any number n of separation parameters. In the present study, we have examined the simultaneous variation of %B, temperature, pH and buffer concentration for the separation of a mixture of substituted benzoic acids.

Single-parameter relationships

Figs. 3–6 show representative plots of sample retention vs. each of the four variables examined in this study: %B, T, pH and buffer concentration. In each case, the solid curves through the data points represent predictions of the appropriate fitting equations (eqns. 3, 5, and 7a–c). Apart from the excellent fit observed in each case, it can be seen that a change in each of these parameters can markedly affect the separation of one or more band-pairs in the sample. That is, whenever two k' plots intersect it signifies that a change in that variable can result in band reversals and marked changes in resolution.

Interaction effects and resulting limits on multi-parameter mapping

The main question in the use of the present resolution-mapping procedure concerns the limits that must be placed on various values of the separation variables
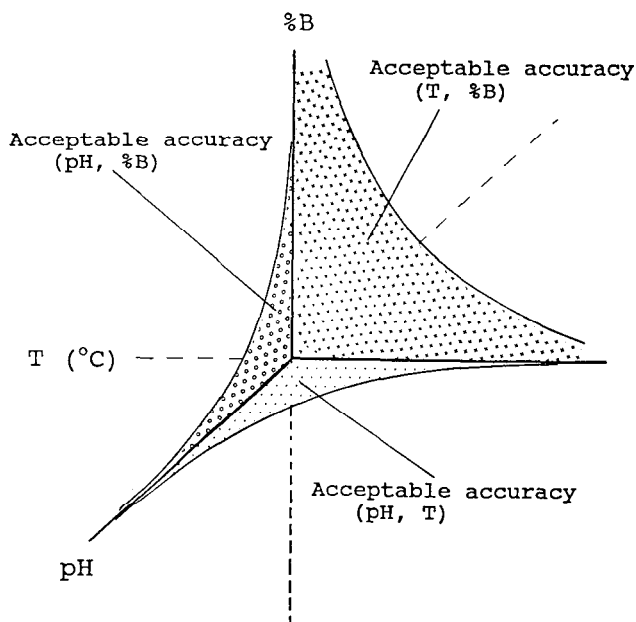
Fig. 2. Illustration of applicability of simplified multi-parameter mapping for prediction of separation when two variables are changed simultaneously (for accuracy in predicted values of α better than $\pm 2\%$).

Fig. 3. Dependence of retention of substituted benzoic acid sample on methanol concentration. Conditions: pH 2.9, 35°C, 25 mM buffer concentration. Solid curves are from eqn. 3; solutes are in the order of Table IV with 2-nitrobenzoic acid at the bottom and 2,6-dimethylbenzoic acid at the top.

Fig. 4. Dependence of retention of substituted benzoic acid sample on temperature. Conditions: pH 2.6, 35% B, 25 mM buffer concentration. Solid curves are from eqn. 5; solutes identified in Table IV.

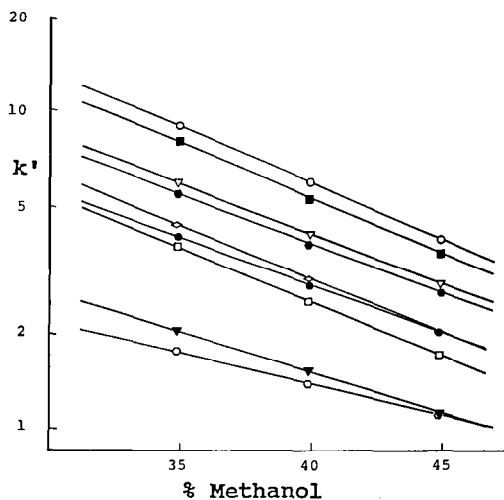during computer simulations, so as to maintain the required accuracy in predicted values of α (as illustrated in Fig. 2). One approach to determining such limits was illustrated in the Theory section (discussion of Tables II and III), for a sample of (mainly) non-ionized compounds reported by Gant et al. [20]. Here we wish to extend these findings to a sample whose components are partially ionized under the conditions of separation, and to include certain other variables (pH, buffer concentration).

Fig. 5. Dependence of retention of representative benzoic acids on pH. Conditions: 35% B, 35°C, 25 mM buffer concentration. Solid curves are from eqns. 7a–c; solutes are (A) 2-nitrobenzoic acid, (E) 3-cyanobenzoic acid and (I) 2,6-dimethylbenzoic acid.

Fig. 6. Dependence of retention of representative benzoic acids on buffer concentration. Conditions: pH 3.2, 40% B and 35°C. Solid curves are from eqn. 8; solutes are in the order of Table IV, with 2-nitrobenzoic acid at the bottom and 2,6-dimethylbenzoic acid at the top.
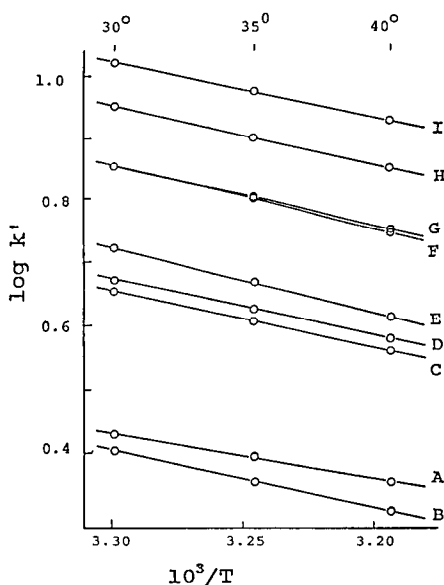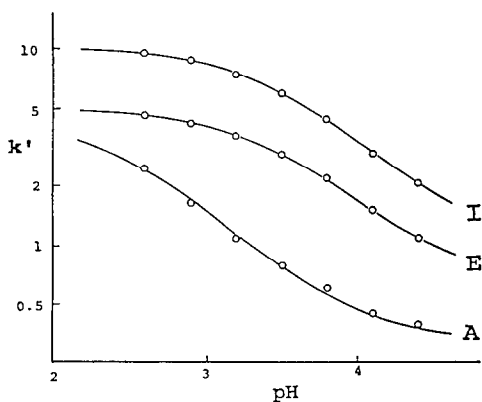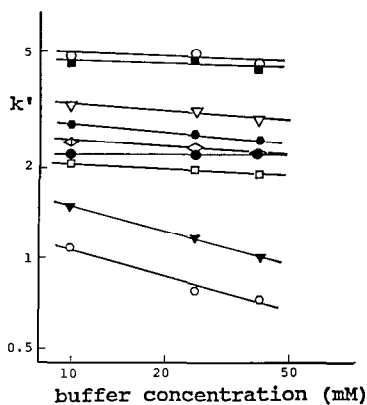
TABLE IV

RETENTION DATA FOR SUBSTITUTED BENZOIC ACIDS AS A FUNCTION OF EXPERIMENTAL CONDITIONS

See Experimental section for other conditions.

Exptl. conditions				Retention times (min)[a]								
pH	%B	T	Buffer	A	B	C	D	E	F	G	H	I
2.6	35	30	25	9.43	9.05	14.11	14.61	16.04	20.87	20.87	25.51	29.40
		35	10	10.22	10.22	12.83	12.90	14.40	18.91	18.91	22.15	26.00
			25	8.91	8.42	12.97	13.44	14.51	18.86	18.86	23.04	26.80
			40	8.14	7.47	12.18	12.78	13.64	17.66	17.66	21.80	25.16
		40	25	8.39	7.81	11.89	12.35	13.11	17.10	16.99	20.78	24.32
	40	30	25	7.94	7.43	10.11	10.86	11.60	15.14	15.14	17.96	20.27
		35	10	8.72	8.72	9.46	9.99	10.70	14.03	14.03	15.85	18.26
			25	7.53	7.00	9.43	10.10	10.65	13.84	13.84	16.39	18.59
			40	6.88	6.25	8.96	9.70	10.12	13.10	13.10	15.68	17.66
		40	25	7.10	6.53	8.75	9.38	9.73	12.56	12.56	14.88	16.99
	45	30	25	6.82	6.30	7.65	8.35	8.76	11.35	11.35	12.95	14.38
		35	10	7.73	7.46	7.73	8.08	8.55	11.12	11.12	12.15	13.82
			25	6.47	5.96	7.19	7.84	8.12	10.43	10.43	11.92	13.28
			40	5.99	5.42	7.04	7.80	7.94	10.14	10.14	11.78	13.04
		40	25	6.11	5.61	6.72	7.48	7.48	9.53	9.53	10.88	12.16
2.9	35	30	25	7.16	8.05	12.83	13.55	14.77	17.51	18.88	24.35	26.87
		35	10	8.08	9.26	12.04	12.50	13.64	16.58	17.64	21.59	24.45
			25	6.88	7.56	11.91	12.58	13.49	16.30	17.23	22.18	24.75
			40	6.34	6.80	11.33	12.08	12.82	15.49	16.29	21.24	23.55
		40	10	7.16	8.28	10.85	11.32	12.12	14.58	15.52	19.33	21.81
			25	6.16	6.84	10.72	11.36	11.96	14.29	15.11	19.76	21.99
			40	5.73	6.23	10.28	10.99	11.46	13.70	14.41	19.10	21.14
	40	30	25	6.20	6.64	9.39	10.20	10.80	12.99	13.85	17.23	18.78
		35	10	7.03	7.69	8.99	9.57	10.19	12.52	13.18	15.50	17.35
			25	5.98	6.30	8.85	9.60	10.03	1..21	12.81	15.90	17.49
			40	5.52	5.70	8.44	9.23	9.55	11.59	12.11	15.24	16.63

TABLE IV *(continued)*

Exptl. conditions				Retention times (min)[a]								
pH	%B	T	Buffer	A	B	C	D	E	F	G	H	I
		40	10	6.28	6.93	8.24	8.78	9.19	11.16	11.73	14.03	15.66
			25	5.40	5.75	8.07	8.80	8.99	10.83	11.34	14.31	15.71
			40	5.04	5.27	7.76	8.64	8.64	10.38	10.84	13.83	15.08
	45	30	25	5.47	5.64	7.19	7.91	8.20	9.86	10.43	12.44	13.44
		35	10	6.30	6.67	7.25	7.80	8.17	10.01	10.47	11.89	13.22
			25	5.29	5.29	6.86	7.60	7.72	9.35	9.75	11.63	12.65
			40	4.95	4.95	6.72	7.56	7.56	9.12	9.50	11.52	12.44
		40	10	5.67	6.05	6.70	7.22	7.44	9.01	9.39	10.83	11.98
			25	4.83	4.98	6.37	7.04	7.04	8.45	8.79	10.62	11.53
			40	4.55	4.55	6.24	6.89	6.89	8.24	8.54	10.50	11.33
3.2	35	30	25	5.26	6.80	10.44	11.32	12.29	12.29	15.15	21.78	21.78
		35	10	5.78	7.46	9.87	10.47	11.30	12.04	14.08	19.01	19.92
			25	5.07	6.36	9.75	10.54	11.20	11.76	13.76	19.63	20.16
			40	4.76	5.82	9.26	10.10	10.59	11.18	12.97	19.00	19.13
		40	25	4.93	6.01	9.22	9.92	10.36	11.15	12.69	18.01	18.88
	40	30	25	4.72	5.71	7.98	8.81	9.22	9.78	11.39	15.78	15.78
		35	10	5.15	6.22	7.61	8.22	8.59	9.45	10.69	13.79	14.51
			25	4.45	5.38	7.51	8.26	8.49	9.20	10.43	14.24	14.68
			40	4.31	4.97	7.18	8.11	8.11	8.80	9.92	13.68	14.01
		40	25	4.43	5.10	7.14	7.93	7.93	8.76	9.69	13.17	13.78
	45	30	25	4.29	4.90	6.32	7.10	7.16	7.77	8.78	11.50	11.69
		35	10	4.73	5.46	6.34	6.88	7.07	7.87	8.72	10.80	11.41
			25	4.16	4.66	6.01	6.67	6.67	7.37	8.13	10.54	10.90
			40	3.98	4.34	5.90	6.56	6.56	7.22	7.95	10.48	10.76
		40	25	4.05	4.46	5.76	6.32	6.32	7.05	7.63	9.86	10.31
2.9	35	35	25	6.71	7.48	11.82	12.49	13.38	16.00	17.03	22.15	24.65
3.2				5.36	6.61	10.27	11.06	11.77	12.67	14.56	20.43	21.37
3.5				4.59	5.97	8.64	9.38	9.98	9.98	12.04	18.07	17.70
3.8				4.09	5.45	7.05	7.60	8.17	7.60	9.62	14.93	13.79
4.1				3.72	4.96	5.58	5.84	6.41	5.84	7.42	10.97	10.01
4.4				3.53	4.60	4.76	4.76	5.38	4.97	6.19	8.18	7.81
3.8	30			4.43	6.29	8.61	9.07	9.07	10.23	11.99	19.23	17.44
	35		10	4.56	6.19	7.47	7.93	8.65	8.25	10.33	15.10	14.44
			40	3.95	5.19	6.88	7.42	7.97	7.42	9.34	14.73	13.52
	40		25	3.74	4.65	5.70	6.26	6.40	6.26	7.52	11.08	10.49
5.2	30		25	3.48	3.86	4.33	3.86	4.83	4.45	5.56	6.28	5.75
	35		10	3.13	3.34	3.61	3.34	3.90	3.76	4.39	4.94	4.50

[a] A = 2-Nitrobenzoic acid; B = phthalic acid; C = impurity; D = 2-fluorobenzoic acid; E = 3-cyanobenzoic acid; F = 2-chlorobenzoic acid; G = 3-nitrobenzoic acid; H = 3-fluorobenzoic acid; 1 = 2,6-dimethylbenzoic acid.

Variation of S, $\bar{B}$ and D (eqns. a–d). Table IV summarizes the experimental data used in the present investigation. Four separation parameters (%B, T, pH and buffer concentration) were varied singly and in combination, so as to allow the study of interaction effects as given by eqns. 9a–c. These data allow the determination of values of S, $\bar{B}$ and D as a function of the other variables, in the same general way as outlined in Table II. The resulting data are summarized in Table V.

As in our example of Table II, the parameters S, $\bar{B}$ and D show both (a) an average variation due to change in the various conditions (T, pH, buffer concen-

TABLE V

SUMMARY OF THE VARIATION OF THE CHROMATOGRAPHIC PARAMETERS S, $\bar{B}$ AND D AS A FUNCTION OF CHANGE IN DIFFERENT EXPERIMENTAL CONDITIONS

Separation parameter	Expt. condition		Variation in parameter	Error in α^a	
	Variable	Change		Calc.	Measured
S	T	$+10°C$	-0.05 ± 0.07	$\pm 0.6\%$	$\pm 0.4\%$
	pH	$+0.6$	-0.21 ± 0.19	$\pm 1.6\%$	$\pm 2.9\%$
	buffer	$+30$ mM	$+0.22 \pm 0.08$	$\pm 0.7\%$	$\pm 0.6\%$
B	pH	$+0.6$	-126 ± 118	$\pm 1.0\%$	$\pm 2.1\%$
	buffer	$+30$ mM	$+49 \pm 19$	$\pm 0.2\%$	$\pm 1.2\%$
D	pH	$+0.6$	$+0.010 \pm 0.033$	$\pm 1.6\%$	$\pm 2.7\%$

^{a} "Calculated" values determined as in Table II (assumes changes of $\pm 5\%$ in B, $\pm 5°C$ on T, ± 0.3 units in pH and ± 15 mM in buffer); "measured" values determined as in Table III or VII.

tration) and (b) scatter. This scatter leads to errors in the prediction of values of α from eqn. 1. The reference run assumed in Tables IV and V is for 40% B, 35°C, pH = 2.9 and a 25 mM buffer concentration. We have used data for $\pm 5\%$ B, $\pm 5°C$, ± 0.3 units in pH and ± 15 mM buffer concentration to determine values of (k_i/k_R) in eqn. 1; the calculated errors in α reported in Table V correspond to changes in each variable by the latter amount ($\pm 5\%$ B, etc.).

Because of the roughly linear change in δS and the other parameters of Table V with the concentration of the second experimental condition ("variable"), it is possible to derive some relationships that predict the total error in a value of α from eqn. 1; these are summarized in Table VI. If the error in α is defined as $\delta\alpha$, then for simultaneous change in three or more variables we have

$$\delta\alpha^2 = \sum^i \delta\alpha_i{}^2 \tag{10}$$

TABLE VI

ERROR IN PREDICTIONS OF α FROM EQN. 1 DUE TO INTERACTION EFFECTS

Expt. variables	Error in α	Maximum change^a	
%B, T	0.00016 (Δ%B) ($\Delta°C$)	11 %B	11°C
%B, pH	0.014 (Δ%B) (ΔpH)	0.3 pH	5°C
%B, buffer	0.004 (Δ%B) ($\Delta\log$ [X])^b	10 %B	$3 \times^c$
T, pH	0.014 (ΔpH) ($\Delta°C$)	0.3 pH	5°C
T, buffer	0.008 ($\Delta\log$ [X]) ($\Delta°C$)	8°C	$2 \times^c$
buffer, pH	0.30 (ΔpH) ($\Delta\log$ [X])	0.25 pH	$1.8 \times^c$

^{a} e.g., a simultaneous change of 11 %B and 11°C is allowed, as is a change of 5.5% B and 22°C, etc.
^{b} [X] is the buffer concentration.
^{c} Indicated change in [X] that is allowed; i.e., 1.8 to 3-fold.

TABLE VII

ERRORS IN α AS A RESULT OF INTERACTION EFFECTS

Comparison of experimental data in Table IV with values from eqn. 1. Errors for Bands C-I only; Bands A and B gave larger, more erratic errors, for reasons which we do not fully understand at present.

Run conditions				Error in α (δα)	
pH	%B	T	Buffer	Found	Eqn. 10[a]
2.6	35	30	25	0.023	0.029
		35	10	0.030	0.036
			25	0.018	0.021
			40	0.030	0.036
		40	25	0.026	0.029
	40	30	25	0.025	0.021
	40	35	10	0.029	0.027
			40	0.034	0.027
		40	25	0.023	0.021
	45	30	25	0.025	0.029
		35	10	0.036	0.036
			25	0.026	0.021
			40	0.044	0.036
		40	25	0.033	0.029
		average difference		±0.004	
2.9	35	30	25	0.005	0.004
		40	25	0.003	0.004
		35	10	0.006	0.006
		40	10	0.015	0.016
		35	40	0.004	0.006
		40	40	0.009	0.015
	40	40	10	0.009	0.012
		40	40	0.019	0.012
	45	30	25	0.004	0.004
		40	25	0.003	0.004
		35	10	0.009	0.006
		40	10	0.014	0.015
		35	40	0.010	0.006
		40	40	0.017	0.015
3.2	35	30	25	0.054	0.029
		35	10	0.031	0.036
			25	0.024	0.021
			40	0.050	0.036
	40	30	25	0.032	0.021
	45	30	25	0.024	0.029

[a] Best fit to eqn. 10.

where $\delta\alpha_i$ is the error from one of the combinations of Table VI. The limits defined in Table VI (for an error in α from eqn. 1 < ±2%) are incorporated into the DryLab MP software. These limits are in the process of further refinement and extension to other separation conditions (see Table I).

Direct measurement of errors in α from eqn. 1. We have also compared experimental values of α with values predicted from eqn. 1 for the various runs in Table
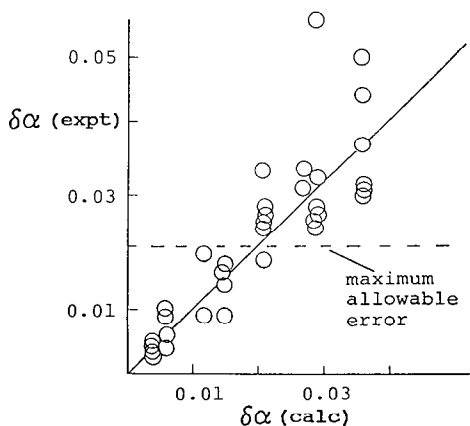
Fig. 7. Comparison of experimental changes in α ($\delta\alpha$) due to interaction effects (simultaneous change of two or more separation conditions) with values predicted from Table VI and eqn. 10.

IV. The resulting average "errors"[a] $\delta\alpha$ are summarized in Table VII. Before analyzing these data, the repeatability of experimental values of α must be addressed. Several replicate runs were carried out for our reference conditions, with the conclusion that values of α were repeatable within about $\pm 0.8\%$ (1 S.D.). About half of this variation appears to be due to small errors in the pH of the mobile phase, which is difficult to reproduce to better than ± 0.02 units. In order to minimize the accumulation of errors from the reference run and the other runs required in the application of eqn. 1 (equivalent to the determination of S, $\bar{B}$, D, etc.), average values of k' were determined from three replicates of the reference run. Similarly, multiple data points and an extended range in each variable were employed in order to maximize the accuracy of values of S, $\bar{B}$, D, etc. In this way the effect of experimental repeatability (error) was minimized, so that resulting determinations of error in eqn. 1 could be directly related to interaction effects. That is, our treatment in Table V was designed to minimize random error contributions —and provide good estimates of $\delta\alpha$ due to interaction effects alone.

Returning to the data in Table VII, these values of $\delta\alpha$ were used to determine the interaction effects as defined in Table V. Table V (last column) summarizes these "measured" values, which are a best fit to the data in Table VII. The corresponding "calculated errors in α" in Table V (next to last column) were determined as described in Table II; these "calculated" and "measured" values should be similar —but not identical. Thus the "measured" values refer to errors in α for the present sample; they therefore depend on the elution order (and values of α) of these compounds. The "calculated" errors in α do not depend on elution order and are therefore less sample specific; they are also less subject to errors in experimental values of retention time.

The "measured" errors in α of Table V, which form the basis of the limit equations of Table VI, were then used to recalculate values of $\delta\alpha$ in Table VII— as a test of (a) the internal consistency of these data and (b) the reliability of eqn. 10. Good

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[a] These "errors" in α (expt. *vs.* eqn. 1) are due to interaction effects.

TABLE VIII

VALUES OF S FOR SOME ALKYL PHTHALATES AS A FUNCTION OF TEMPERATURE AND COLUMN

Data calculated from study of ref. 21.

Solute[a]	Values of S[b]		
	Column A[c]		Column B[c]
	35°	60°	35°
C_1	2.24	2.12	2.21
C_2	2.56	2.49	2.55
C_3	2.95	2.78	2.90
C_4	3.50	3.48	3.40
C_5	3.96	3.94	3.86
Average δS[d]	-0.08 ± 0.06		-0.06 ± 0.03

    [a] Refers to alkyl chainlength; e.g., C_1 is dimethyl phthalate.
    [b] Calculated for 60 and 75% acetonitrile–water for indicated columns (A, B) and temperatures (35 and 60°C).
    [c] Both columns are C_8 for reversed-phase; column A has a pore diameter of 6 nm and column B has a pore diameter of 15 nm; the surface area of column B is therefore smaller.
    [d] Calculated as in Table II ("error in S") relative to column A at 35°C.

overall agreement is seen, as summarized in Fig. 7. Here values of $\delta\alpha$ (expt) are the "found" values of Table VII, while $\delta\alpha$ (calc) are values of $\delta\alpha$ obtained from eqn. 10 and the "measured" errors in α from Table V.

Data for dialkyl phthalates

    Data dealing with the dependence of S on temperature are reviewed in Tables II and III for a non-ionized sample taken from the literature [20]. Similar data have been reported for a series of dialkyl phthalates [21], separated at different temperatures on different columns. These data are summarized in Table VIII.

    It is interesting to compare the values of δS for the three cases examined here, normalized to a change in T of 25°C: substituted benzenes (Table II), -0.41 ± 0.05, benzoic acids (Table V), -0.12 ± 0.17 and alkyl phthalates (Table VIII), -0.08 ± 0.06. The phthalates show less effect of temperature on the average value of δS, but the variability of δS (which determines errors in α from eqn. 1) is comparable to the substituted benzenes. This suggests that other non-ionized solutes will exhibit a similar dependence of S on temperature. The limits of Table VI (derived for the benzoic acids in Table IV) are thus overly conservative when applied to non-ionic solutes.

    Values of S (at 35°C) are seen to be similar for the two columns (A and B) of Table VIII, implying that values of S (and $\bar{B}$, D, etc.) determined for one column will be applicable to another column. This observation has important implications, which are considered further in the following paper [22].

pH mapping and the determination of values of K_a, $k°$ and k^+

    We have noted that the accurate prediction of retention as a function of pH

(based on the measurement of values of K_a, k° and $k^\pm$) requires care in the selection of three runs (including the reference run) with varying pH. We have found it difficult to make up mobile phases within better than ± 0.02 units of the desired pH, and other errors in the determination of values of k' are unavoidable. As a consequence, the application of eqns. 7a–c for the purposes of measuring values of K_a, k° and $k^\pm$ requires that the difference in pH values be fairly large; *i.e.*, a pH range of ≈ 1 unit or greater, corresponding to pH values of *e.g.*, 4, 4.5 and 5 in the three runs. The required range in pH values must be even greater, when the range selected does not bracket the pK_a values of the sample.

However, the use of differences in pH > 0.5 units between adjacent runs can create other problems. Changes in band spacing and relative band areas as pH is varied are well documented, making peak tracking (required in all retention mapping procedures) more difficult. This problem becomes more serious, as the sample becomes more complex, and as the change in pH between runs is increased. Ideally, it would be preferable to use smaller differences in pH; *e.g.*, < 0.3 units. Thus, some compromise between peak tracking and the accurate determination of K_a, k° and $k^\pm$ for each solute is necessary. We will address this issue elsewhere, in conjunction with a new approach to peak tracking which is underway in our laboratory [23].

CONCLUSIONS

A new procedure for developing and optimizing HPLC separations has been described, based on limited-range multi-parameter mapping. Any number of experimental parameters can be varied simultaneously, and only a few experimental runs are required for computer simulations of separation as a function of these experimental conditions. Only the primary effects of each experimental variable on separation are considered, but errors due to interaction effects are predictable. This allows the restriction of multi-parameter mapping to combinations of conditions that yield acceptable errors ($< \pm 2\%$) in predicted values of α. Errors in retention time will generally be larger ($< 25\%$), but this will have little effect on predicted values of resolution (which is of primary importance in method development).

In the present study, we examined the effects of changing %B, temperature, pH and buffer concentration, for the separation of a 9-component substituted benzoic acid sample. In addition, more limited data for other samples from the literature were included. Based on the present study it appears that this approach can provide accurate predictions of separation (especially values of α and resolution) over a fairly wide range of conditions, while requiring only a small number of experimental runs. This, in turn, leads to a number of practical applications of the related software that we have developed (DryLab MP)— as described in the following paper.

SYMBOLS (PARTS I AND II)

Reference to equations in the papers in this series is identified by use of I- or II-, *e.g.*, eqn. I-1 refers to eqn. 1 in Part I.

A	constant in eqn. I-5;
A	weaker solvent in a mobile phase A/B

A_i, A_j	values of A (eqn. I-5) for solutes i and j
B	strong solvent in a mobile phase A/B
$\bar{B}$	coefficient measuring dependence of retention on temperature for a given solute (eqn. I-5)
$\bar{B}_i, \bar{B}_j$	values of $\bar{B}$ for solutes i and j
C, D	constants in eqn. I-8 for a given solute
D_1, D_2	values of D in eqn. I-8 for different mobile phase additives and a given solute
F	mobile phase flow-rate
F^-	fraction of an acidic compound which is ionized under the conditions of separation
F^+	fraction of a basic compound which is ionized under the conditions of separation
$F^\pm$	fraction of an acidic or basic compound which is ionized under the conditions of separation
$[H^+]$	concentration of hydrogen ion in the mobile phase
k'	solute capacity factor
k_i	value of k' for some change in the separation parameter i, other conditions remaining the same as for the reference run (eqn. I-1)
k_R	value of k' for reference conditions
k_w	value of k' for water as mobile phase (eqn. I-3)
k_{wi}, k_{wj}	values of k_w for solutes i and j
k°	value of k' for an acidic or basic solute in the non-ionized form
$k^\pm$	value of k' for an acidic or basic solute in the ionized form
K_a	acid dissociation constant; equal to 10^{-pK_a}
MP	multi-parameter
N	column plate number
N_0	value of N for large k'
R_s	resolution of two adjacent bands
S	solute parameter, equal to $-\,\mathrm{d}\,(\log k')/\mathrm{d}\varphi$
S_i, S_j	values of S for solutes i and j
t_R	solute retention time (min)
T	temperature (K)
W	bandwidth (eqn. II-2, min)
W_{ec}	contribution to W from extra-column effects and the dependence of N on k' (eqn. II-2, min)
W_0	bandwidth due to the column alone (eqn. II-1, min)
X	a mobile phase additive (buffer, ion-pair reagent, amine modifier, etc.)
$[X_1], [X_2], \dots$	concentrations of different mobile phase additives 1, 2, ...
$[X]$	concentration of X in the mobile phase separation factor, equal to ratio of values of k' for two adjacent bands
δS	a change in S due to change in some other variable(s)
α	separation factor for two solutes
$\delta\alpha$	an error in a predicted value of α due to the neglect of interaction effects
$\delta\alpha_i$	a contribution to $\delta\alpha$ from a specific interaction effect (eqn. I-10)
$\Delta\bar{B}$	difference in $\bar{B}$ values for adjacent solute bands i and j; equal to $\bar{B}_i - \bar{B}_j$
ΔS	difference in S values for two adjacent solutes i and j; equal to $S_i - S_j$
φ	volume fraction of strong solvent B in mobile phase A/B, equal %B/100

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