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REVIEW

DRYLAB® COMPUTER SIMULATION FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD DEVELOPMENT

I. ISOCRATIC ELUTION

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1. INTRODUCTION

Method development in high-performance liquid chromatography (HPLC) consists of various steps, among which are the selection of experimental conditions for adequate separation, *e.g.*, baseline resolution of all bands or resolution $R_s > 1.5$. At the same time, other goals are often important:

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(1) reasonable run time, *e.g.*, < 10 min;
(2) acceptable operating pressure, *e.g.*, < 2000 p.s.i.;
(3) minimum peak volume for maximum signal-to-noise ratio (S/N);
(4) rugged method, insensitive to minor changes in separation conditions;
(5) minimum time and effort spent on method development.

An experienced chromatographer usually balances these various goals according to the nature of the sample and the intended use of the final HPLC procedure.

The need for efficient HPLC method development has led to a number of proposals, sometimes referred to as "optimization" strategies (for reviews, see refs. 1–3). Most of these approaches involve various means for computer-assisted retention mapping. In some instances, sample resolution (R_s for the poorest-resolved band pair) is determined as a function of mobile phase composition. Alternatively, more complicated measures of separation (chromatographic "response functions") may be used as indices of chromatographic performance. Although commercial software of this type has been available for almost a decade, it appears that relatively few practising chromatographers routinely make use of these procedures at present.

More recently we have developed software (DryLab®) based on a different approach to computer-assisted HPLC method development (see ref. 3 for a summary of our philosophy). This procedure, which can be referred to as "computer simulation", attempts to mimic the strategy followed by experienced chromatographers, but uses the computer to reduce the required time and effort. Some critical aspects of computer simulation are reviewed in Table 1. Our use of computer simulation can be summarized as follows:

(1) Initially rank the various separation variables that affect retention and band spacing, *e.g.*, concentration and type of solvents in the mobile phase, mobile phase pH, concentration and type of mobile-phase additives, column type.

TABLE 1

CHARACTERISTIC FEATURES OF COMPUTER SIMULATION (DRYLAB)

Feature	*Consequences*
Theory based	DryLab minimizes the number of experimental runs required and increases the total number of separation variables that can be simulated (*e.g.*, column conditions, gradient conditions)
User driven	User can select the best approach to method development based on sample characteristics and separation goals; decisions on how to proceed (at each step) are made by the user; no "black box" optimization takes place
Can duplicate slower experimental procedures	User employs computer simulation in the same way as experimental trial-and-error method development; chromatograms can be requested, and separation data can be displayed in various ways by the computer
Other additional features	Adjustment of column plate number to match experimental chromatogram(s); overlay of gradient at end of column; plots of resolution *vs.* separation conditions; summary tables of various kinds
Facilitates systematic method development	Previous experience in HPLC method development[3] plus computer simulation can be used to arrive at suitable separation conditions with a minimum of effort and time

(2) Carry out a small number (usually two or three) of experimental runs in which only one mobile-phase variable is changed; this is followed by further "experiments" (simulations) with a computer, making use of (a) simulated chromatograms, (b) tabulated data for a simulated run and/or (c) tabular or graphical summaries for several runs.

(3) Select the "best" separation conditions based on computer simulation plus experimental verification of this result; if the resulting separation meets the goals of the HPLC method, no further change in the separation conditions is required.

(4) For further improvements in the separation, carry out additional experimental runs (usually two) for a new separation variable (*e.g.*, a different strong solvent, varying pH, a different column); repeat the computer simulation as in (2) and select the conditions for the best overall separation.

This procedure can be continued (and additional separation variables mapped) until an adequate separation is achieved. Any number of separation variables can be explored, and any combination of these variables can be simulated and studied. The present approach is therefore open-ended (to address the needs of "difficult" samples), but less difficult separations will require relatively little effort and only a small number of experimental runs (the user can stop whenever an adequate result is achieved).

Computer simulation as developed by us emphasizes those experimental variables that have a primary effect on sample retention and band spacing. However, this software also permits computer simulation of so-called "column conditions" for a given sample, *i.e.*, change in column dimensions, particle size and flow-rate can be simulated without additional data. In this way, the column plate number can be optimized for a given application, so as to provide a good compromise among different separation goals such as resolution, run time, pressure and peak volume.

2. THEORY AND BACKGROUND

Computer simulation (DryLab) is based on simple (but reliable) theory for the following relationships: (1) dependence of retention on mobile phase composition; (2) dependence of column plate number on experimental conditions; (3) interrelationship of isocratic and gradient retention; and (4) predictability of gradient retention as a function of gradient conditions.

2.1. Retention vs. mobile phase composition

There is no adequately precise theory that allows *ab initio* predictions of HPLC retention as a function of change in the mobile phase. However, numerous studies (see review in ref. 4) have established that the empirical relationship

$$\log k' = \log k_w - S\varphi \tag{1}$$

is relatively accurate for the variation of strong solvent concentration (%B or φ) in reversed-phase separations. Here, k' refers to the capacity factor of a given solute for a mobile phase of composition φ, the volume fraction of the strong solvent in the mobile phase, and k_w and S are constants that are characteristic of the strong solvent and solute. Minor deviations from eqn. 1 are common, but their impact on pre-

dictions of k' as a function of φ has been thoroughly studied[5,6]. The resulting errors in values of k' are small for either interpolation or modest extrapolation.

Eqn. 1 can be generalized to the form

$$\log k' = A - Bx \tag{2}$$

where x is now any of the common variables used to adjust HPLC retention, *e.g.*, temperature, pH, additive or buffer concentration or fraction of one mobile phase in a mixture of any two mobile phases. Eqn. 2 assumes that $\log k'$ varies linearly with x; for the sample variables just enumerated (temperature, pH, etc.), this relationship is neither fundamental not precise; however, as long as the range in x is restricted within certain limits, eqn. 2 can be used for estimates of retention as a function of x with adequate precision (Table 2). We shall illustrate this further.

2.2. Plate number as a function of experimental conditions

The plate number N can be expressed with reasonable accuracy by the Knox equation[1], for so-called "ideal" conditions. Estimates of solute diffusion coefficient as a function of mobile phase viscosity and temperature and solute molecular weight are also required[8], but predictive relationships are well established for both small molecules[1] and large biomolecules[9]. Various studies[7,9] suggest that predicted bandwidths (and resolution) will agree with experimental values to within ± 10–20% in the absence of certain complicating factors ("non-ideal" separation).

An initial experimental run can also be compared with predictions of bandwidth as above. If the experimental value is significantly in error, the chromatographer has two options. First, for experimental values that are low (*e.g.*, by a factor of three or more, often accompanied by band tailing), it is advisable to explore possible causes of this discrepancy[10]. It may be possible to increase the experimental value of N by changing the separation conditions (different pH, addition of amine modifier, increase in temperature, etc.). Alternatively, it is possible to determine the ratio of predicted *vs.* experimental N values and use this "correction factor" to adjust predicted values of N. DryLab software offers this option to the user, so that reliable predictions of bandwidth become possible for moderately "non-ideal" systems.

2.3. Isocratic vs. gradient separation

The use of eqn. 1 as a simplifying assumption allows a rigorous calculation of retention in gradient elution for any combination of gradient conditions[9,11–13]. Addi-

TABLE 2

EQN. 2 FOR PREDICTIONS OF RETENTION IN HPLC

Variable	*Allowed range in values of X*
Temperature	30–40°C range, *e.g.*, 25 60°C
pH	1–2 pH units[a]
Mixture of organic solvents	0–50% of second solvent in organic solvent mixture[a]
Mobile phase additives	Factor of 10 in concentration

[a] May require correction, based on a third experimental run.

tionally, it is possible to derive experimental values of k_w and S (required in predictions of retention for both isocratic or gradient runs) from two initial isocratic or gradient runs. The use of computer simulation for developing a gradient elution method is described in Part II[14].

2.4. Systematic method development

Computer simulation is best used in conjunction with an overall approach to systematic method development[3]; that is, it is essential to properly prioritize the choice of separation variables for study by computer simulation. For this reason, we recommend the following method development strategies.

2.4.1. Reversed-phase HPLC. Carry out initial runs with acetonitrile–water (B–A) mobile phases, buffered at pH 3.5 if acids or bases are present in the sample. Adjust the %B for adequate sample retention ($1 < k' < 20$ for all bands) and fine-tune %B within this range for maximum resolution. If the resolution is inadequate at this point, substitute methanol for acetonitrile and repeat this procedure. If resolution is still inadequate, substitute tetrahydrofuran for methanol and repeat the procedure. Computer simulation combined with estimates of equi-eluotropic strength (p. 32 in ref. 3) can minimize the number of experimental runs required.

Further improvements in separation can be achieved by mapping resolution *vs.* mobile phase composition for both mixtures of the above three organic solvents[15] (B) and change in solvent strength (%B)[16]. Where appropriate, other separation variables (*e.g.*, pH or temperature) can be changed and separation mapped as a function of experimental conditions (using computer simulation).

2.4.2. Ion-pair HPLC. Use a similar procedure as for reversed-phase HPLC, except select methanol as organic solvent and vary the mobile phase pH and the concentration of the ion-pair reagent[3].

2.4.3. Normal-phase HPLC. Use a similar procedure as for reversed-phase HPLC, except choose different strong solvents, *e.g.*, methylene chloride, methyl *tert.*-butyl ether (MTBE) and either acetonitrile or ethyl acetate[3].

2.4.4. Gradient elution. Use a similar procedure as for the above isocratic methods, but explore changes in gradient conditions first. Multi-segment gradients are especially useful for adjusting overall band spacing and resolution[13,17,18] (see Part II[14]).

3. EXPERIMENTAL AND SOFTWARE

3.1. Equipment and materials

Separations reported for the first time (here or in the Part II[14]) were carried out on a Beckman/Altex System Gold HPLC system (Beckman Instruments, San Ramon, CA, U.S.A.); its dwell volume was 2.3 ml. A variable-wavelength UV detector is part of this system; wavelengths used are indicated in the text. Solvents and reagents were of HPLC grade. Water was purified with a Milli-Q system (Millipore, Milford, MA, U.S.A.).

3.2. Software

Computer simulations were carried out by means of DryLab I (this paper) or DryLab G (Part II[14]) software (LC Resources, Lafayette, CA, U.S.A.).

4. REPRESENTATIVE EXAMPLES OF METHOD DEVELOPMENT BASED ON COMPUTER SIMULATIONS

The following illustrations are selected to show various features of computer simulation.

4.1. Mixture of six steroids

The application of computer simulation for this sample has been described briefly elsewhere[19]. Method development was begun with a 25 × 0.46 cm I.D. Zorbax C_8 (5 μm) and a flow-rate of 2.0 ml/min. Acetonitrile (B) was selected as the organic solvent because of its lower viscosity and UV absorbance (see Discussion in ref. 3). Two gradient runs were carried out: 5–100% B in times of 15 and 45 min, as shown in Fig. 1A and B. Examination of these chromatograms shows only five bands in each run, whereas the sample contains six components. As it appears that the same two components overlap in each run (comparison of band areas), it is unlikely that this sample can be separated with any acetonitrile–water mixture as mobile phase. A change in organic solvent is therefore indicated.

4.1.1. Methanol as solvent: optimum %B. Methanol is usually the next best choice[3], and two gradient runs were repeated with this organic solvent: 5–100% B in 15 and 45 min (Fig. 1C and D). These runs show six bands in each chromatogram and are therefore more promising. The experimental conditions, retention times and band area from the runs in Fig. 1C and D were entered into the computer, as summarized in Table 3 (A). Computer simulation could now be used to study how separation varies with the percentage of methanol in the mobile phase (%B).

The first requirement is to maintain the sample retention within an appropriate range, *e.g.*, $1 < k' < 20$ for all sample bands. It is therefore expedient to request a survey of retention *vs.* mobile phase composition, as shown in Table 3 (B) for eleven mobile phases which range from 0 to 100% methanol; the last column provides the k' range of the sample and it can be seen that mobile phases with 40–60% B have the desired solvent strength.

We can next request a relative resolution map, *i.e.*, a plot of resolution R_s for the poorest separated band pair *vs.* %B, for a 10 000-plate column. Fig. 2A shows such a map for a range of %B that covers the desired k' range (1–20). It is seen that maximum resolution occurs for an intermediate methanol concentration (about 50% B). A final decision regarding the best value of %B can now be obtained be requesting information on separation *vs.* %B in 1% increments, as shown in Table 3 (C); the second column shows a maximum resolution of $R_s = 2.01$ for 48% methanol.

The mobile phase that gives maximum resolution is not always the best choice, however. Method ruggedness is an equally important consideration. In a routine laboratory that makes use of an HPLC method, variability of different kinds is always present. For example, Table 3 (C) indicates that an error in the mobile phase formulation of ±3% could lead to a decrease in resolution of about 25% ($R_s = 1.55$ for 45% B). If our mobile phase choice were 50% B (instead of 48% B), however, the corresponding maximum decrease in resolution would be only 7%. An added advantage of choosing 50% B is that the run time is only 15 min *vs.* 19 min for 48% B [Table 3 (C)].

Other studies have shown[20] that column-to-column variations in retention and

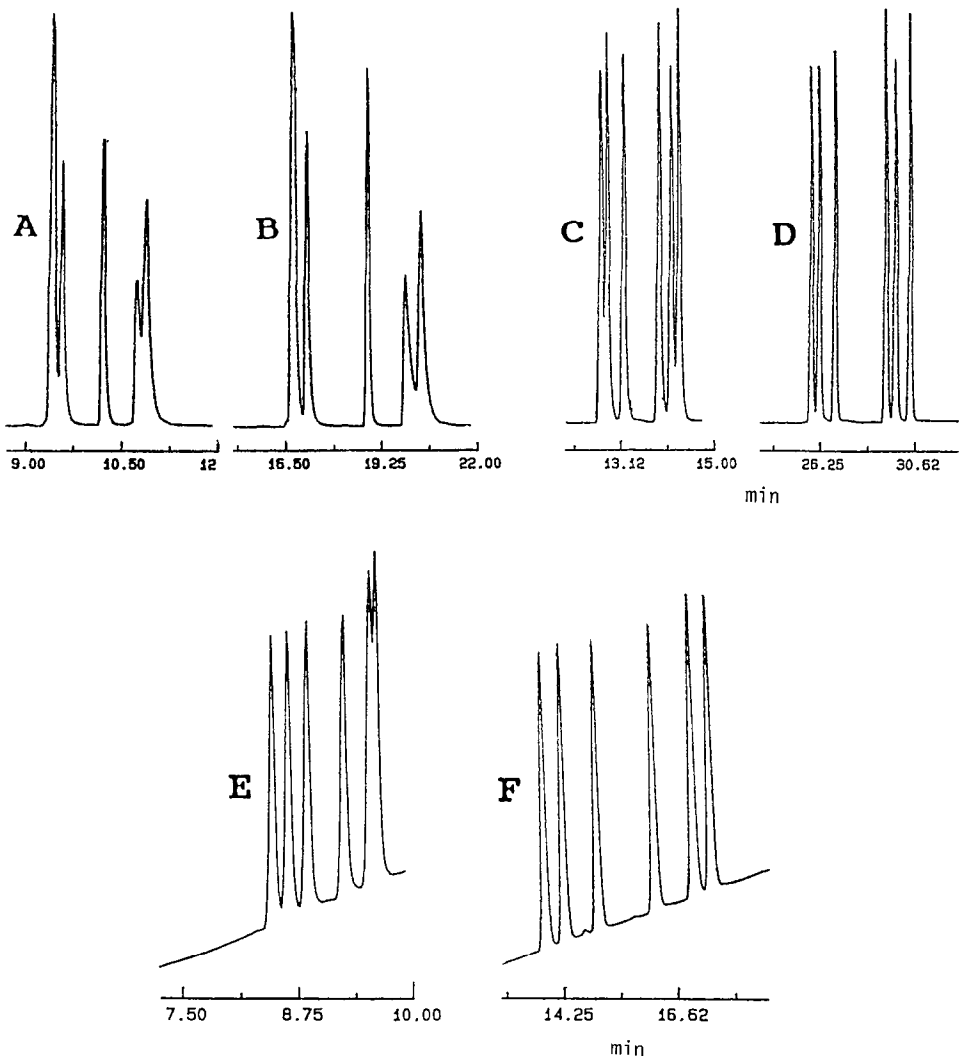

Fig. 1. Experimental runs for steroid sample. Conditions: 25 × 0.46 cm I.D. Zorbax C_8 column; 5–100% B gradients; temperature, 35°C; flow-rate, 2 ml/min; detection at 254 nm. Sample: prednisone, cortisone, hydrocortisone, dexamethasone, corticosterone; cortexolone. See ref. 19 for details. Solvent B and gradient time vary: (A) acetonitrile, 15 min; (B) acetonitrile, 45 min; (C) methanol, 15 min; (D) methanol, 45 min; (E) THF, 15 min; (F) THF, 45 min.

separation can be adjusted to some extent by changing %B. This suggests that a procedure that is relatively insensitive to variation in %B (50% methanol for the present sample) will also be less sensitive to changes in retention from column to column. This is another reason for choosing 50% methanol as the mobile phase. The final separation with 50% methanol and other conditions unchanged is shown in Fig. 3A. The actual resolution (bands 1 and 2) is $R_s = 1.3$ with a run time of 15 min, *i.e.*, the experimental column plate number is less than 10 000.

TABLE 3

COMPUTER-SIMULATION DATA FOR STEROID SAMPLE

(A) DryLab input data; (B) computer-simulation summary, k' range (and other results) as a function of %B; (C) as in (B) for 1% increments in %B.

System — *Parameters*

A

System variables

Dwell volume (ml)	5.50
Column length (cm)	25.0
Column diameter (cm)	0.46
Flow-rate (ml/min)	2.00
Starting %B	5.0
Final %B	100.0
Gradient time, 1st run (min)	15.0
Gradient time, 2nd run (min)	45.0

Retention entries:
number of bands = 6

Band	t_R *(min)*		*Area*
	Run 1	*Run 2*	
1	12.71	25.83	36.00
2	12.84	26.20	39.00
3	13.18	26.82	37.00
4	13.89	29.29	39.00
5	14.13	29.72	36.00
6	14.29	30.38	41.00

B

%B	R_s *(10 K)*	α	*Band pair*	*Run time (min)*	k' *range*
0	1.78	1.07	1, 2	4954	1228–3874
10	1.92	1.08	1, 2	1534	403–1199
20	0.43	1.02	3, 1	476	133–371
30	0.14	1.01	2, 3	148	40–115
40	0.68	1.03	4, 5	47	12–36[a]
50	1.94	1.10	1, 2	15	3.4–11[a]
60	1.35	1.11	1, 2	5.6	1.0–3.4[a]
70	0.66	1.12	1, 2	2.6	0.3–1.1
80	0.17	1.03	5, 6	1.7	0.1–0.3
90	0.04	1.02	6, 5	1.4	0.0–0.1
100	0.03	1.14	1, 2	1.3	0.0–0.0

C

%B	R_s *(10 K)*	α	*Band pair*	*Run time (min)*	k' *range*
45	1.55	1.07	4, 5	27	6.3–20
46	1.72	1.08	4, 5	24	5.6–18
47	1.88	1.08	4, 5	21	5.0–16
48	2.01	1.10	1, 2	19	4.4–14
49	1.98	1.10	1, 2	17	3.9–12
50	1.94	1.10	1, 2	15	3.4–11
51	1.90	1.10	1, 2	14	3.0–9.8
52	1.85	1.11	1, 2	12	2.7–8.7
53	1.80	1.11	1, 2	11	2.4–7.7
54	1.75	1.11	1, 2	10	2.1–6.9
55	1.69	1.11	1, 2	9.1	1.9–6.1

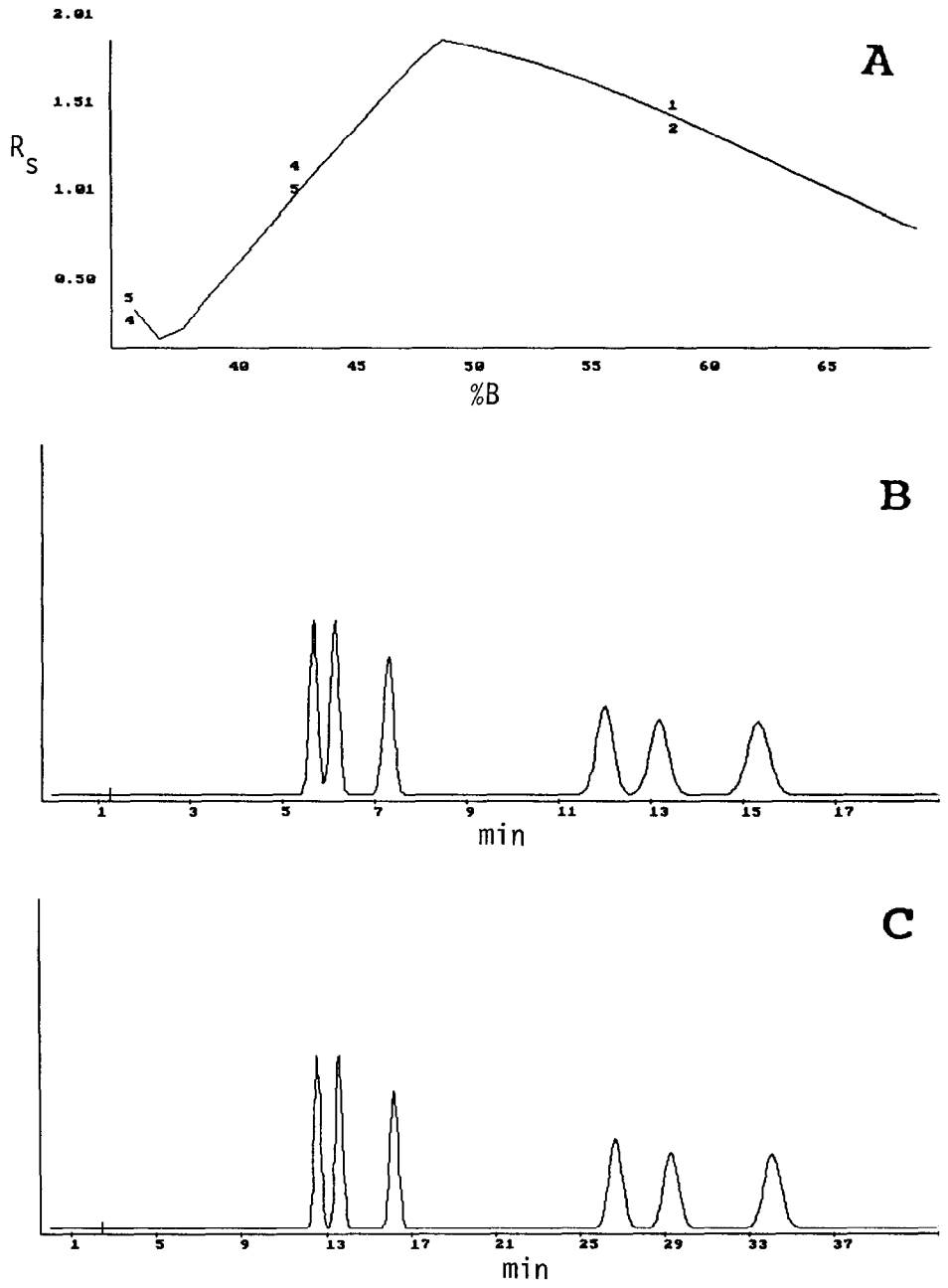

Fig. 2. Computer-simulation results for steroid sample. (A) Relative resolution map (N = 10 000); (B) chromatogram for methanol–water (50:50) (N = 4500), other conditions as in Fig. 1; (C) same as (B), except flow-rate 0.9 ml/min. Numbers in (A) (*e.g.*, 4/5, 1/2) denote poorest-resolved band pair for a given %B.

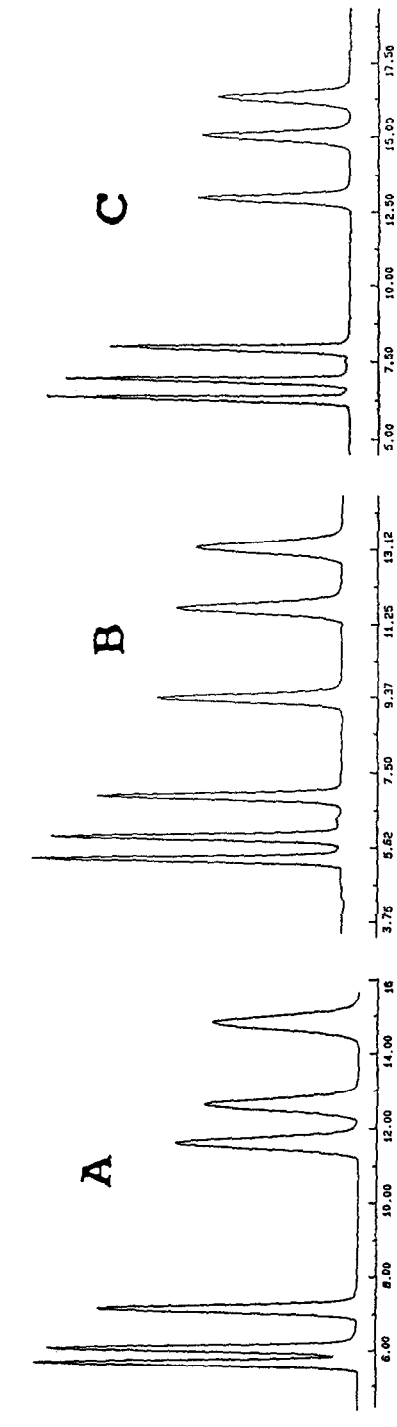

Fig. 3. Experimental separations of steroid sample under optimized conditions. (A) methanol–water (50:50); (B) THF–water (24:76); (C) acetonitrile–THF–water (11:12:77). Other conditions as in Fig. 1.

4.1.2. Methanol as solvent: optimum column conditions. The separation in Fig. 3A is marginal in several respects. Accurate measurement of band size (for quantitative analysis) is favored by baseline resolution between adjacent bands ($R_s > 1.5$). The resolution in Fig. 3A is only $R_s = 1.3$. Generally, it is desirable to have a run time of < 10 min, compared with 15 min in Fig. 3A. Finally, the operating pressure observed for this separation was 4600 p.s.i., which is too large. Pressures of <2000 p.s.i. should generally be the goal.

DryLab can be used to predict separation as a function of column conditions (length, particle size, flow-rate). This is achieved by first estimating the column plate number, N, and if necessary correcting this value on the basis of an experimental run. DryLab predicted a resolution of $R_s = 1.6$ for the separation in Fig. 3A, compared with the actual value of 1.3. This corresponds to $N = 7200$ (predicted) *vs.* 4500 (actual), that is, the column is less efficient than expected for this sample, for various reasons[a]. We can adjust for this difference, so that computer predictions match the experimental runs more closely. The resulting simulation (same conditions as in Fig. 3A) is shown in Fig. 2B; it agrees closely with the experimental chromatogram (Fig. 3A) for the same conditions.

Now the flow-rate and column length can be varied (computer simulations) in a trial-and-error effort to achieve adequate resolution (a value of $R_s = 1.7$ was the goal), a pressure less than 2000 p.s.i. and a run time as short as possible. The best conditions (25-cm column, 0.9 ml/min) gave the chromatogram in Fig. 2C. However, the 34-min run time is excessive. The use of a 3-μm packing was studied next (computer simulations). A 12-cm column (available as 4 + 8-cm lengths in series) lowered the run time to 21 min, but this is still too long for routine use.

4.1.3. Tetrahydrofuran (THF) as solvent. The preceding study (five experimental runs plus 30 min of computer simulation) suggests that another solvent should be tried. THF is usually our last choice, because it is easily oxidized and less convenient to use. Two gradient runs were carried out (5–100% THF, 15 and 45 min), as shown in Fig. 1E and F. Six bands are seen in each chromatogram, and the data from these two runs were entered into the computer. Similar simulations as described above (methanol as solvent) were then repeated. Fig. 4A shows the relative resolution map for THF as solvent, indicating maximum resolution for 24% B. The predicted resolution for these separation conditions was $R_s = 2.4$, compared with an actual resolution of 2.1 (predicted $N = 8200$; actual $N = 6100$). Adjustment of the predicted N value then gave the simulated chromatogram in Fig. 4B. The latter agrees well with the experimental chromatogram in Fig. 3B (same conditions). Further variation of column conditions gave the separation in Fig. 4C (15-cm column, 1.8 ml/min), which exhibits acceptable resolution ($R_s = 1.7$) and pressure (1950 p.s.i.) and a reasonable run time (9 min).

For the present steroid sample, the final HPLC method required eight experimental runs. The total time involved (including computer simulations) was about 1 day. This represents more effort than will usually be required for samples that contain ten or fewer components. The reason is that some samples will be adequately separated with the first or second choice of organic solvent (acetonitrile or methanol). Thus,

[a] Error in the DryLab prediction is one possibility. Alternatively, the column may have been less well packed or have deteriorated in use, or the nominal particle diameter may differ from the actual diameter.

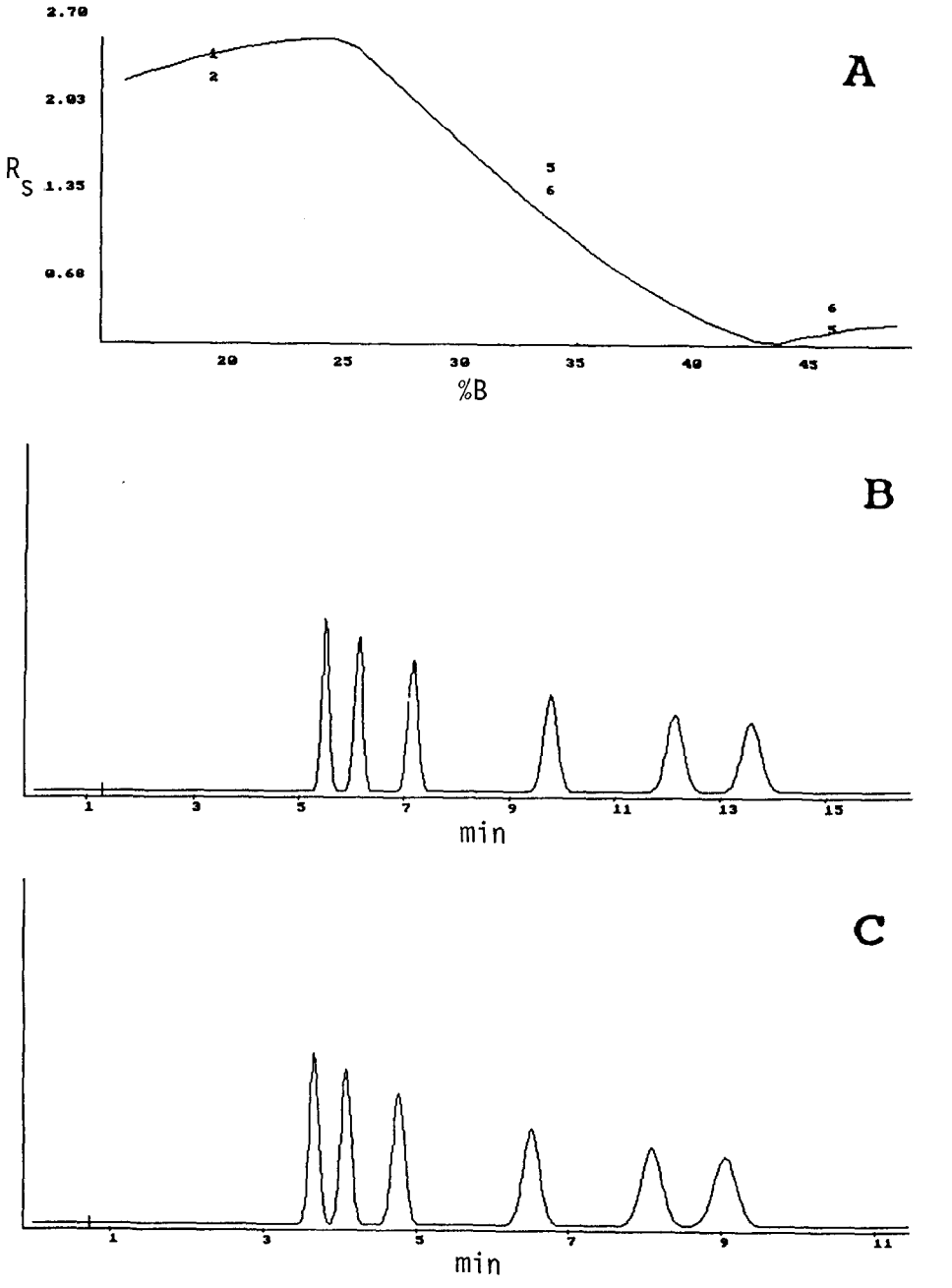

Fig. 4. Computer simulations for separation of steroid sample with THF as solvent B. (A) Relative resolution map (N = 8200); (B) predicted chromatogram for THF–water (24:76); (C) same as (B), except 15-cm column, flow-rate 1.8 ml/min. Other conditions as in Fig. 1.

in a previous study[19] of four representative samples, two were best separated with acetonitrile, one with methanol and one with THF. See also the following example of seven substituted benzenes.

4.2. Mixture of seven substituted benzenes

Wright *et al.*[21] described the separation of a seven-component test sample that was selected to test the efficacy of different computer-assisted approaches to HPLC method development. They found that in this particular instance, the so-called "simplex lattice" procedure (similar to the procedure in ref. 15) was superior to the "sequential simplex" approach[22]. Each of these method-development schemes required a relatively large number of experimental runs: 20 for the sequential simplex procedure and more than 50 for the simplex lattice approach.

As with the steroid sample, we began with two acetonitrile–water gradient runs: 5–95%B in 15 and 45 min (Fig. 5). Data from these initial runs was entered and computer simulation was begun. A survey of retention *vs.* %B (10% increments) was requested, as shown in Table 4. These data (last column) suggest a narrow range of composition (30–40% acetonitrile) such that $1 < k' < 20$ for all bands. A resolution map was next selected that overlapped this optimum k′ range. This is shown in Fig. 6A.

The map in Fig. 6A suggests that mobile phases with 30–40% B cannot provide adequate resolution of the sample ($R_s < 0.6$). However, if we are willing to accept $k' < 1$ for the initial band, excellent resolution ($R_s \approx 2.5$) is possible for a mobile phase with about 55%B. Depending on the nature of the sample, *e.g.*, the likelihood of impurity bands, the extent of baseline distortion near t_0, etc., the choice of 55% acetonitrile as mobile phase might be appropriate. Alternatively, gradient elution might be the preferred option (we pursue this further in Part II[14]).

Fig. 6B shows the predicted chromatogram for a mobile phase of acetonitrile–water (55:45) and Fig. 6C shows the experimental chromatogram that was obtained under the same conditions. The latter separation is quite acceptable, and there is good agreement between the predicted and measured chromatograms.

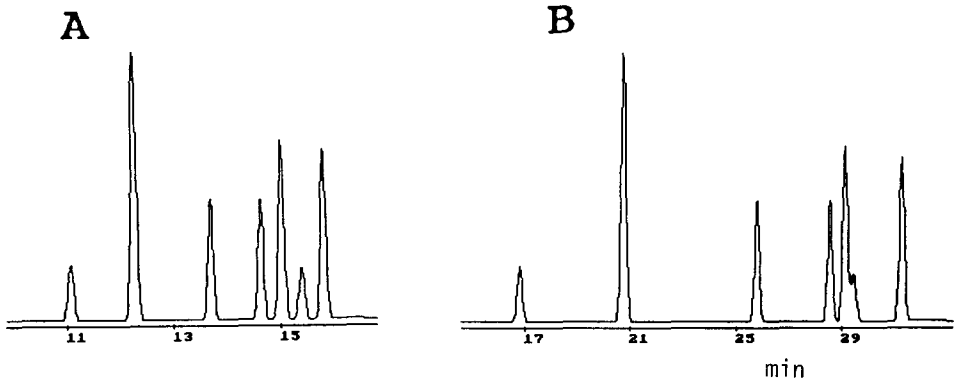

Fig. 5. Experimental chromatograms for seven-component substituted benzene sample (reconstructed data). Conditions: 25 × 0.46 cm I.D. Zorbax Rx column (5 μm); temperature 30°C; flow-rate, 1 ml/min; detection at 230 nm. Sample as in Table 3. Gradient: 5–95% acetonitrile–water in (A) 15 min and (B) 45 min.

TABLE 4

COMPUTER SIMULATION SUMMARY FOR SUBSTITUTED BENZENE SAMPLE IN FIG. 5 (ISOCRATIC SEPARATION, DRYLAB I)

%B	R_s *(10 K)*	α	*Band pair*	*Run time (min)*	*k' range*
0	1.50	1.06	7, 4	819	20–319
10	0.74	1.03	3, 6	334	9.5–130
20	2.65	1.11	5, 4	145	4.6–56
30	0.32	1.01	5, 4	64	2.2–24
40	0.64	1.03	5, 6	29	1.0–10
50	2.56	1.14	5, 6	14	0.5–4.5
60	1.66	1.36	1, 2	7.5	0.2–1.9
70	0.58	1.23	1, 2	4.7	0.1–0.8
80	0.15	1.11	1, 2	3.5	0.1–0.4
90	0.01	1.01	1, 2	3.0	0.0–0.2
100	0.00	1.02	3, 2	2.7	0.0–0.1

4.2.1. Band identification. Method development as described above can be applied to both known and unknown samples. For known samples, the individual bands in the final chromatogram can be identified by injecting standards (as described in ref. 22 for the same sample). However, this would mean an additional seven runs for the present sample. A simpler expedient is to prepare a new sample with different concentrations of each component and to carry out just one additional run for the purposes of peak identification. This approach was followed here, as shown by the chromatogram in Fig. 6D.

As the concentrations of each component in the samples in Fig. 6C and D are known, it is possible to calculate the concentration ratio for each sample (and each chromatogram). This concentration ratio should be the same as the band ratios in each run. Therefore, peak identification can be effected simply by matching the band-area ratios in each run with the sample concentration ratios. This comparison, summarized in Table 5, permits the following assignments in the chromatograms in Fig. 6C and D: (1) benzyl alcohol, (2) *p*-cresol, (3) *n*-propyl hydroxybenzoate, (4) *n*-butyl hydroxybenzoate, (5) diethyl phthalate, (6) toluene and (7) benzophenone.

The development of a method for this sample is seen to have required only four experimental runs, including the extra run that allows all bands in the chromatogram to be identified. This contrasts with the steroid sample, where eight experimental runs were necessary (nine, if individual bands required identification). On average, samples containing less than about a dozen components will usually require about half a dozen experimental runs plus 1 h of computer time when computer simulation is employed.

5. MAPPING SEPARATION AS A FUNCTION OF OTHER VARIABLES

DryLab uses eqn. 2 to linearize plots of retention *vs.* %B. However, this relationship can also provide a reasonable fit for other separation conditions (pH, temperature, mixtures of two organic solvents, etc.), at least over some restricted range in

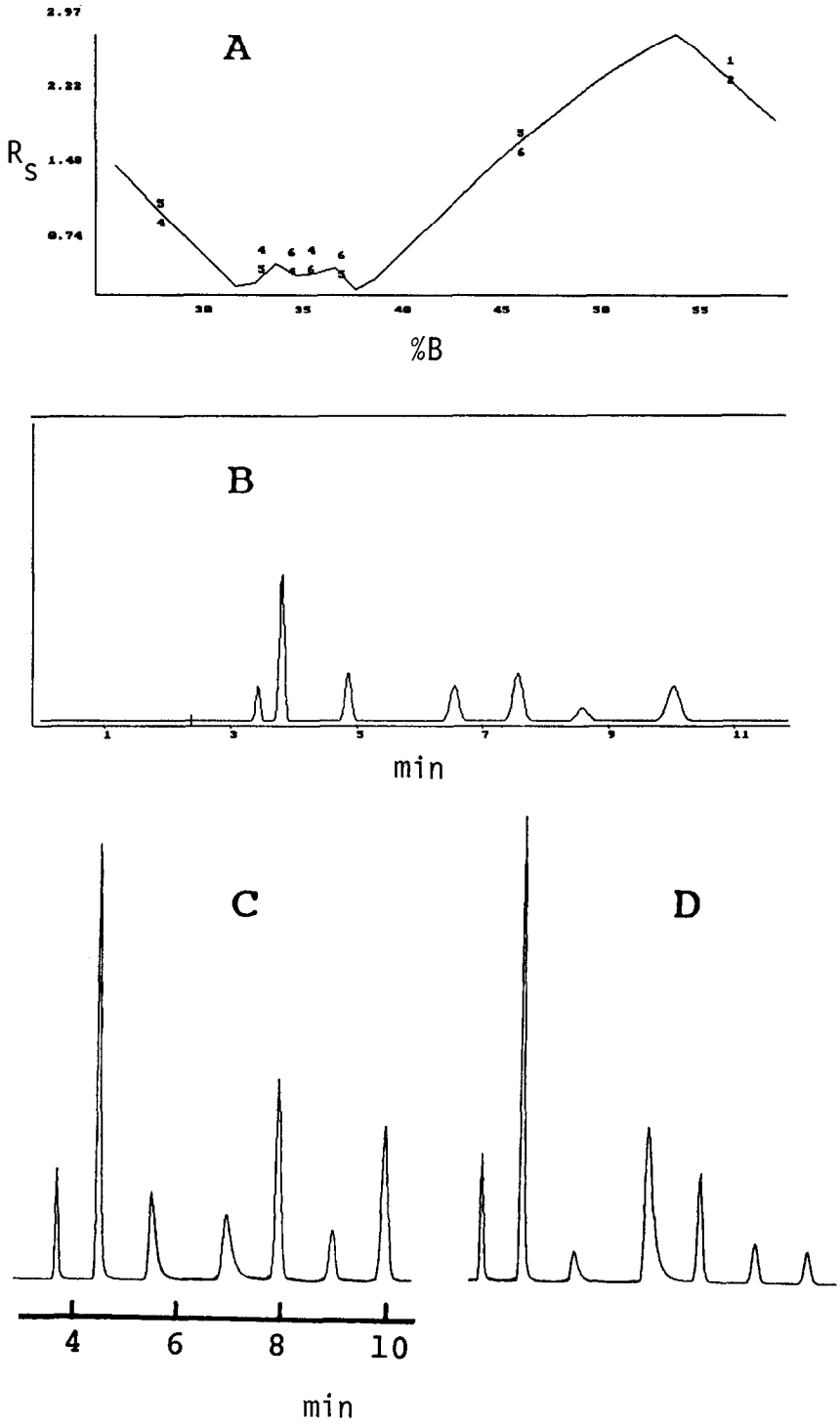

Fig. 6. Method development for substituted benzene sample in Fig. 5. (A) relative resolution map (N = 10 000); (B) predicted chromatogram for acetonitrile–water (55:45) (N = 10 080, value from DryLab); (C) and (D) experimental runs for conditions in (B), different samples (see Table 3).

TABLE 5

PEAK IDENTIFICATION FOR SUBSTITUTED BENZENE SAMPLE IN FIG. 6 BY MEANS OF AREA RATIOS

Sample		*Chromatogram*	
Compound	*Concentration ratio*[a] *(sample 2/sample 1)*	*Band No.*[b]	*Area ratio*[b] *(run 2/run 1)*
Toluene	1	1	1.7
Benzyl alcohol	2	2	1.4
p-Cresol	1.5	3	0.46
Diethyl phthalate	0.75	4	2.7
Benzophenone	0.25	5	0.73
n-Propyl hydroxybenzoate	0.50	6	0.99
n-Butyl hydroxybenzoate	3.0	7	0.27

[a] Ratio of solute concentrations in samples used for run 1 (Fig. 6C) and run 2 (Fig. 6D), respectively.
[b] Band area ratio for run 2 *vs.* run 1; bands numbered in order of elution.

the condition being varied (see Table 2). This means that we can use computer simulation to map separation *versus* any condition that affects retention. We shall illustrate the process here for ternary mobile phases made up from two binary mobile phases: acetonitrile–water (52:48) and THF-water (39:61).

The following data for a nine-component substituted naphthalene sample were taken from ref. 15. DryLab I allows the use of either gradient or isocratic runs as input; in the following example, isocratic data were used. Fig. 7 shows reconstructed chromatograms for two runs using (A) acetonitrile–water (52:48) and (B) THF–water (39:61). Only seven distinct bands can be seen in each run, meaning that there are two pairs of overlapping bands in each run (marked with asterisks). For the following simulations, the second mobile phase (containing 39% THF) is taken as the B solvent. This means that the mobile phase for run A in Fig. 7 can be expressed as 0% B, whereas the mobile phase for run B is considered to be 100% B. The run conditions and retention data for the two runs in Fig. 7 were then used for computer simulation.

In the present example, the k' range of the sample does not change much with %B, can be seen in Fig. 7. Therefore, the first step was to examine an extended resolution map for the separation, as shown in Fig. 8; this consists of three maps (N = 5000, 35% B range each). It is seen that maximum resolution is predicted for a mobile phase composed of 47% B, *i.e.*, 47% of the THF–water (39:61) mobile phase plus 53% of the acetonitrile–water (52:48) mobile phase. The simulated chromatogram for this separation is shown in Fig. 9A and the (reconstructed) experimental run is shown in Fig. 9B. The experimental run exhibits a resolution of R_s = 1.3, which is an improvement over the two initial runs in Fig. 7 (R_s = 0.0). However, there are substantial differences between the predicted and experimental chromatograms in Fig. 9, because eqn. 2 is only a rough approximation over the range 0–100% B (see Table 2).

We can obtain a better approximation by using the experimental run for 47% B (Fig. 9B) to replace either the 0% or 100% B runs for computer simulation (second iteration). If we use 0% and 47% B runs as computer inputs, we obtain the resolution

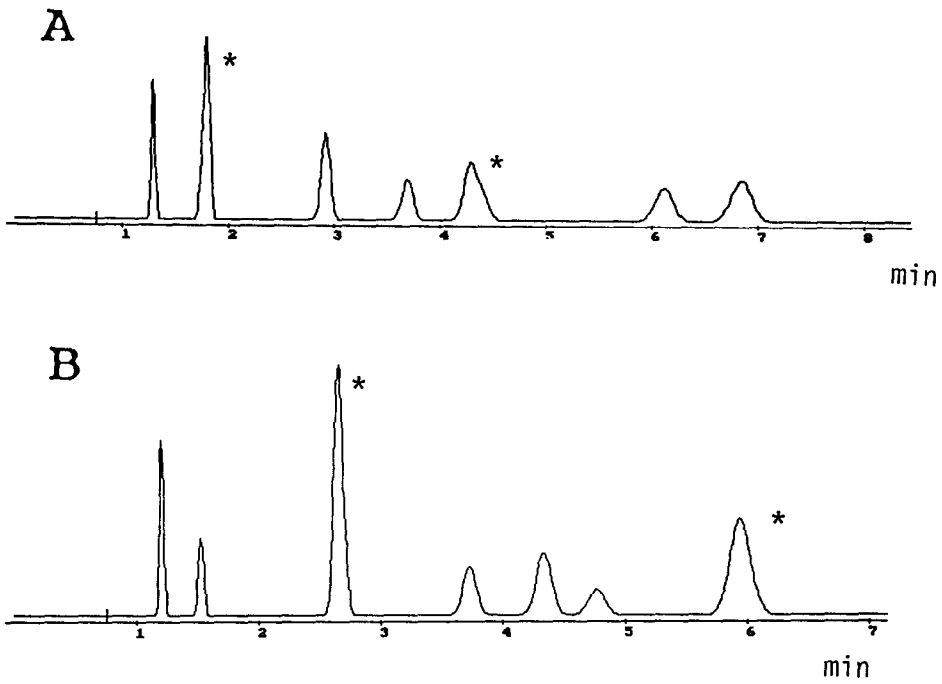

Fig. 7. Reconstructed experimental chromatograms for nine-component substituted naphthalene sample from ref. 15. Conditions: 15-cm Zorbax C_8 column; temperature 25°C; flow-rate 1 ml/min. (A) acetonitrile–water (52:48); (B) THF–water (39:61). Overlapping bands are marked with asterisks.

map in Fig. 10A. Likewise, if we use 47% and 100% B as inputs, we obtain the map in Fig. 10B. Together these provide a better picture of how resolution varies with %B than does the map in Fig. 8. This iterative approach to resolution mapping is similar to the approach described by Schoenmakers *et al.*[23].

From Fig. 10B it can be seen that maximum resolution occurs for a mobile phase containing 64% B. Fig. 10C shows the predicted separation for this mobile phase (R_s = 1.5) and Fig. 10D shows an experimental separation for similar conditions. The chromatograms in Fig. 10C and D are in reasonably close agreement, and it is clear that our use of computer simulation was able to locate this optimum condition with only three experimental runs.

We have used DryLab I to optimize resolution using other variables[24]. So far, few problems have been encountered with variables such as pH, temperature, buffer concentration and ion-pair reagent concentration as long as the approach used here is followed and the ranges in Table 2 are not exceeded.

6. COMPUTER SIMULATION *VERSUS* ALTERNATIVE APPROACHES TO HPLC METHOD DEVELOPMENT

There are now a large number of means for computer-assisted HPLC method development, as outlined in refs. 1–3. In most instances, the experimental protocol is reasonably complicated and a considerable number of experimental runs are re-

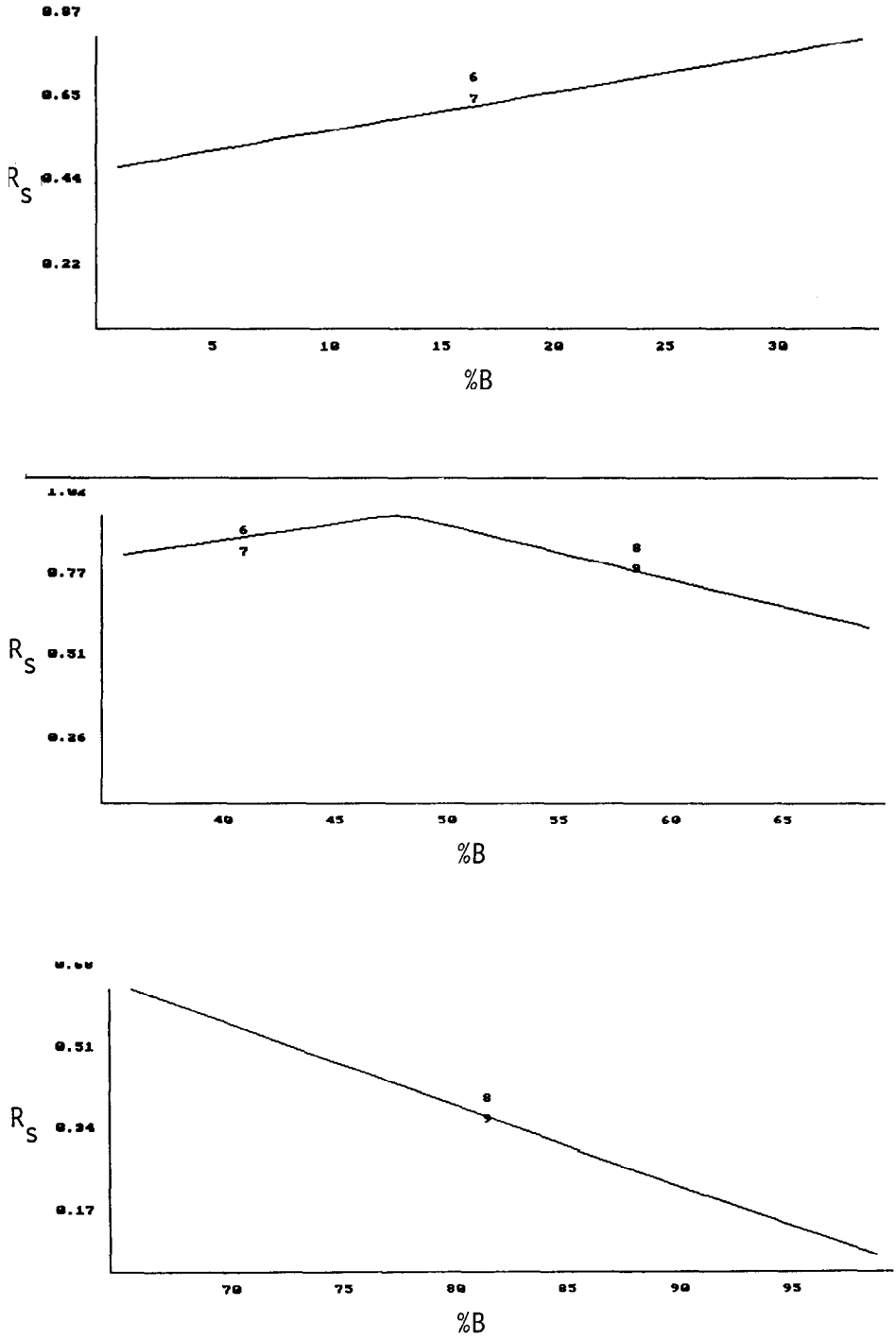

Fig. 8. Extended resolution maps (N = 5000) for substituted naphthalene sample; based on two runs in Fig. 7 as input to DryLab.

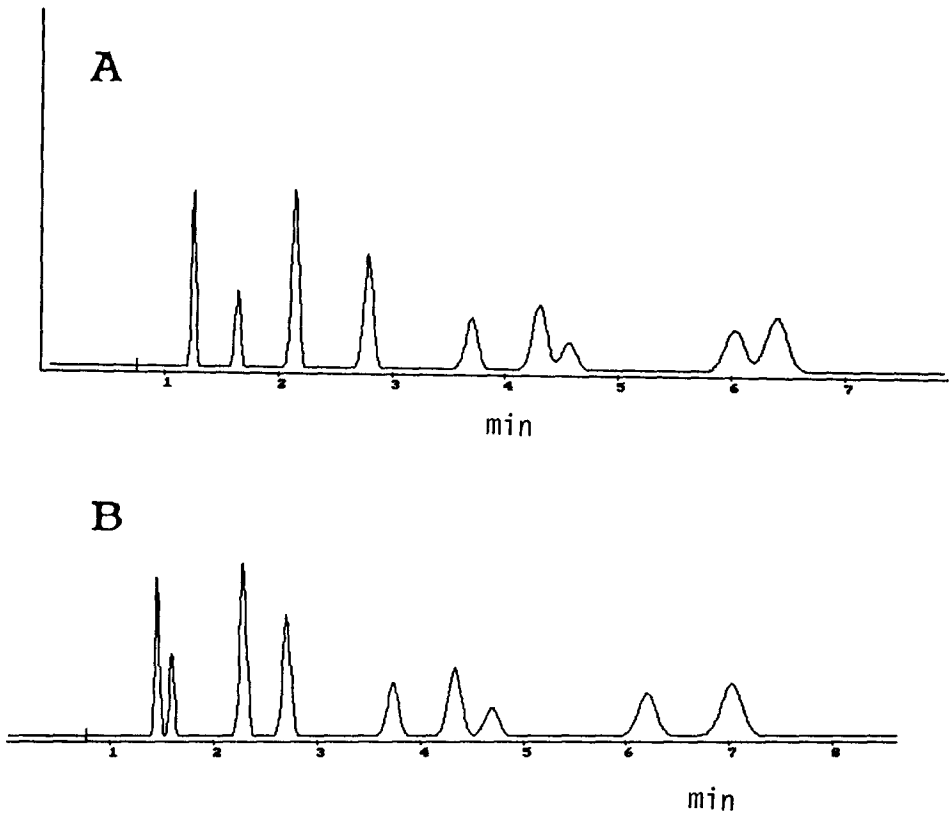

Fig. 9. Semi-optimized separation of substituted naphthalene sample; first approximation. Conditions as in Fig. 7 unless indicated otherwise. (A) Predicted chromatogram; (B) actual (reconstructed) chromatogram. Mobile phase: (THF–water, 39:61)–(acetonitrile–water, 52:48) (47:53) = THF–acetonitrile–water (18.3:27.6:54.1).

quired. Special software is generally necessary, and often this is available only as part of a commercial HPLC system. For these and other reasons, many workers have avoided computer-assisted method development altogether. It is therefore useful to compare the capabilities of a simple approach such as that offered by DryLab with some of these more sophisticated (and potentially more powerful) procedures.

It should be noted that computer simulation as we have used it relies mainly on the changes in band spacing that occur as a result of changes in solvent strength, *i.e.*, mobile phase %B. Until a few years ago, most chromatographers assumed that band spacing normally does *not* change much when %B is varied. Recent work (*e.g.*, refs. 13, 17–19 and 25–27) has conclusively demonstrated that optimization of solvent strength is a powerful tool for optimizing band spacing and resolution for a wide range of sample types. At the same time, this approach to method development is much more easily adapted to computer-assisted method development, and it avoids many of the problems faced by other procedures (see below).

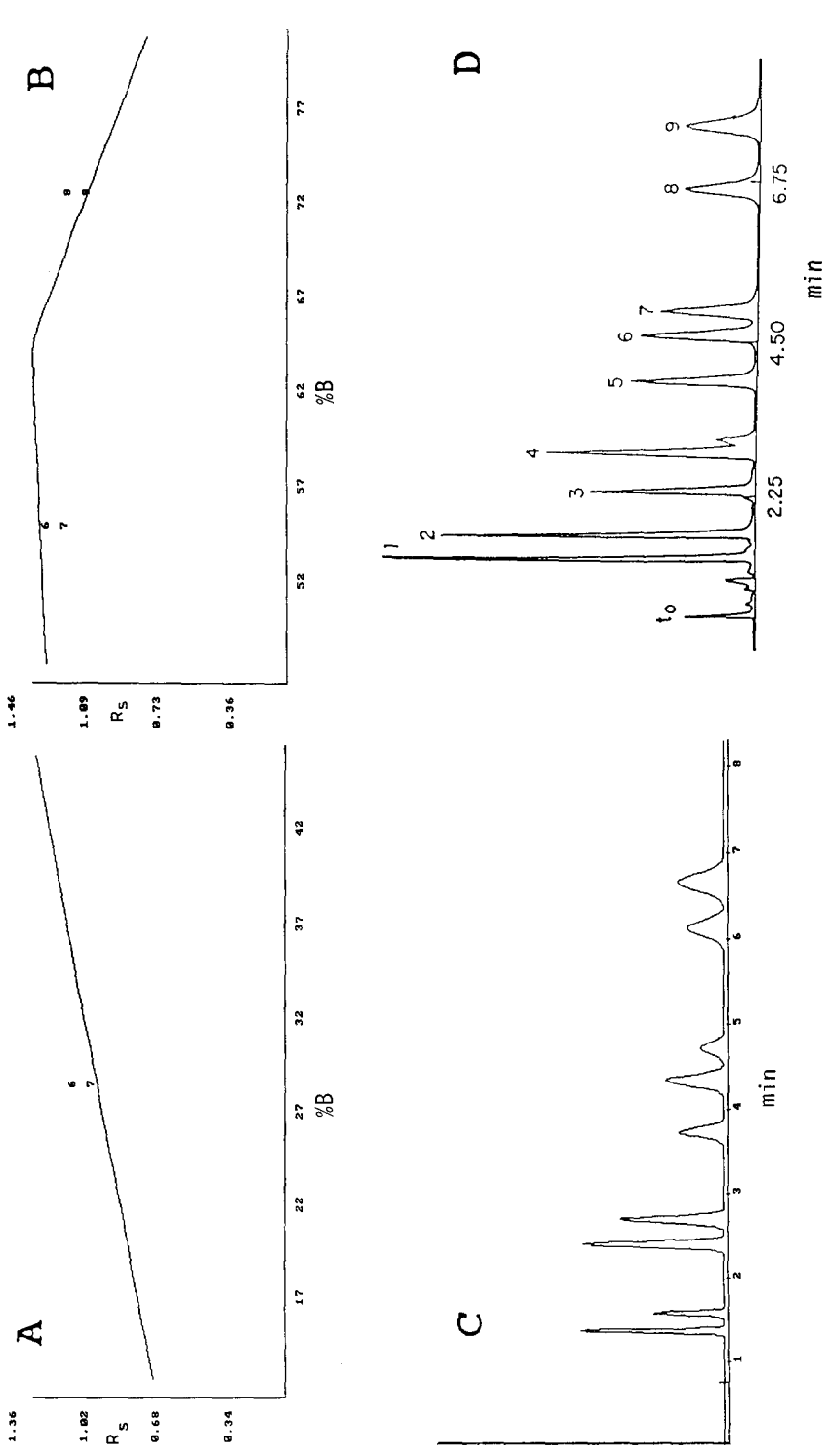

Fig. 10. Final optimized separation of substituted naphthalene sample; second approximation. Conditions as in Fig. 7 unless indicated otherwise. (A) and (B) extended resolution maps (N = 5000) based on three experimental runs; (C) predicted optimum separation for 64% mixture of two starting mobile phases (19% acetonitrile plus 25% THF); (D) experimental optimum for 68% mixture of starting mobile phases (17% acetonitrile plus 26% THF).

6.1. Six-component steroid sample

We described above the development by computer simulation of a method for this sample. Fig. 3B shows the best separation obtained in this way. The Kirkland–Glajch simplex-lattice method[15] was also used for this sample. This involves mapping resolution *versus* all possible compositions of water, methanol, acetonitrile and THF in the mobile phase for some fixed run time (fixed solvent strength or average k' values). The best separation using the latter approach gave the optimum separation in Fig. 3C. The resolution is essentially identical for the two runs, but the DryLab method is 4 min shorter. The simplex-lattice method required about 50 experimental runs[a] for this sample, compared with eight runs for computer simulation.

6.2. Substituted benzene sample

The separation of this sample based on computer simulation was discussed above. The best separation (only four experimental runs required) is shown in Fig. 11C. The similar application of simplex-lattice and sequential-simplex procedures[21] for method development is shown in Fig. 11A and B, respectively[b]. The resolution in chromatogram C is seen to be substantially superior to that in A and especially B. In addition, a much greater experimental effort (20–50 runs) was required in each of the last two approaches.

The last two examples (Figs. 3 and 11) suggest that computer simulation will often be preferred for HPLC method development over manual (trial-and-error) procedures or other computer-assisted schemes. This hypothesis is further confirmed by our experience summarized elsewhere[19] with other such samples. We believe that this will prove to be true in most instances involving "average" samples, *i.e.*, containing fewer than about ten components. More complex samples, and those where pH optimization is the preferred approach, may benefit from the use of more elaborate retention-mapping schemes.

6.3. Peak tracking

A major problem in all computer-assisted method-development procedures is the necessity to match bands between two runs. That is, if compound A comprises band 1 of run 1, it must be known where compound A elutes in run 2 (or run 3, 4, etc.). This is illustrated in the two runs in Fig. 7. There are nine compounds in each chromatogram, and the bands in each run that contain a given compound must be known before computer simulation can begin. In this instance, compounds A–I can be assigned as follows:

acetonitrile–water (52:48): A < B + C < D < E < F + G < H < I

THF–water (39:61): A < B < C + D < E < F < G < H + I

[a] This could have been reduced to 15–20 runs by taking advantage of the peak-matching procedure of Table V.

[b] It should be noted that a different column was used for computer simulation in this instance. Therefore, we cannot say that this example "proves" the superiority of computer simulation over the simplex-lattice and sequential-simplex approaches. Rather, it should be emphasized that in this instance and others that involve simple sample mixtures, computer simulation has consistently given an acceptable separation with a small experimental effort.

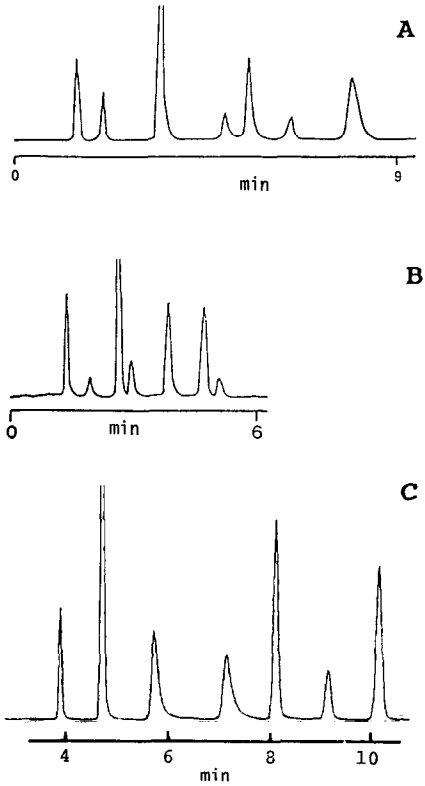

Fig. 11. Separations of seven-component substituted benzene sample; comparison of results of different computer-assisted method-development approaches. (A) Simplex lattice; (B) sequential simplex; (C) computer simulation. Chromatograms (A) and (B) from ref. 21.

In other instances, band reversals will be present, further complicating the untangling of each chromatogram.

Peak tracking has so far not proved to be a major problem in computer simulation (*i.e.*, the DryLab approach). The approach that we recommend is to involve the chromatographer in carrying out peak assignments. Often a simple visual comparison of two chromatograms (*e.g.*, Figs. 1 and 5) is all that is required for peak matching, especially when only the solvent strength is varied. If there is any question at this point, a comparison of band areas (expressed as area%) usually suffices to resolve any doubts. Finally, it is always possible that two bands of similar size may change places in the chromatogram (from one run to the next), without being recognized. In these instances, it is usually sufficient to carry out a third (intermediate) run and compare the predicted with the actual chromatogram. If the two chromatograms agree, then all bands have been assigned correctly.

This approach is illustrated in Fig. 12 for the separation of a ten-component nitroaromatic sample. Examination of Fig. 12A and B for acetonitrile–water (40:60) and (60:40), respectively, shows that a number of changes in band position have occurred as a result of this change in %B. Matching of bands between the two runs is

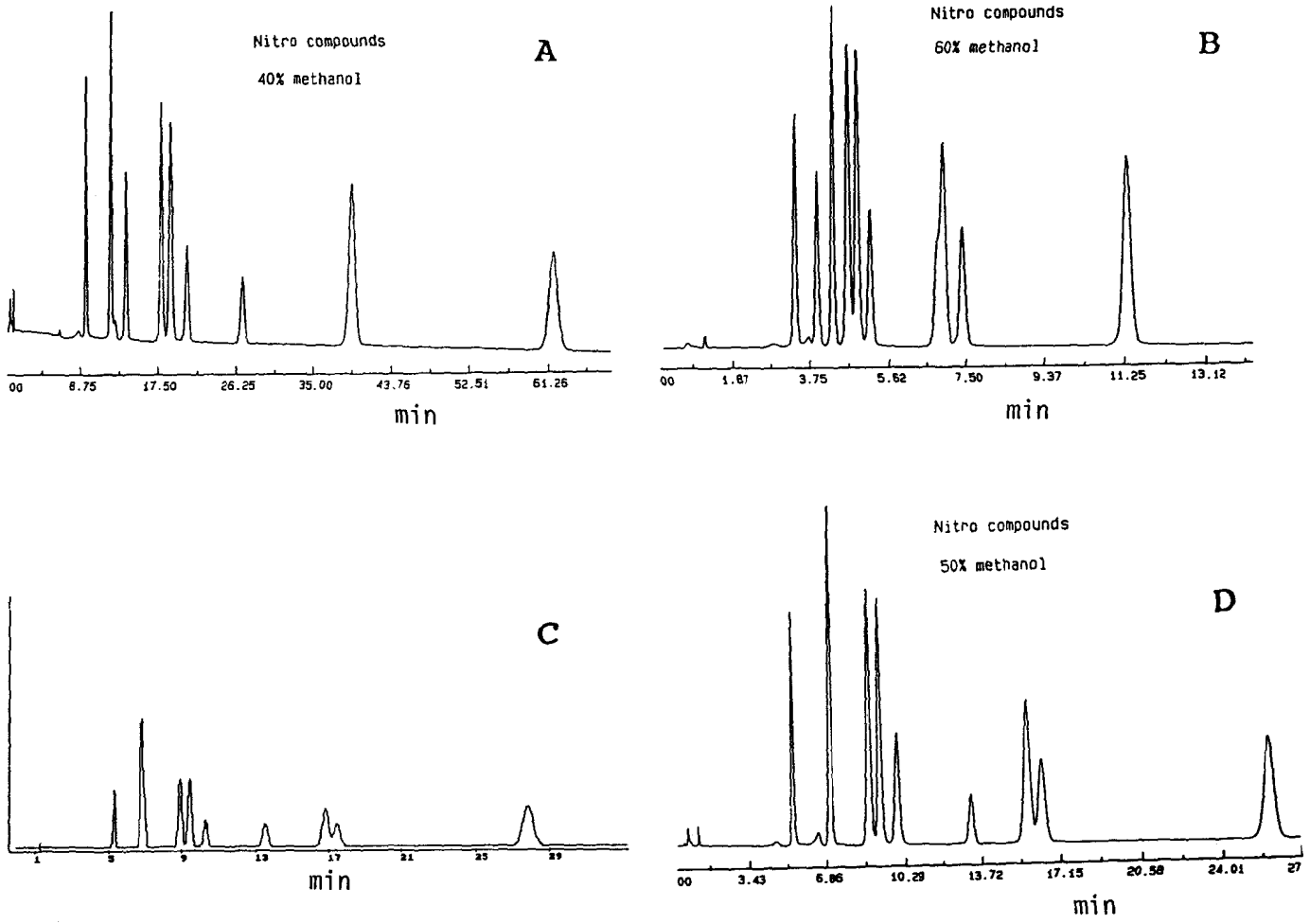

Fig. 12. Separation of ten-component nitroaromatic sample as a function of methanol concentration in water. Conditions: 25 × 0.46 cm I.D. Zorbax C_8 column; temperature 35°C; flow-rate, 2 ml/min. (A) (B) and (D) experimental runs for indicated %B; data from ref. 28; (C) simulated run based on 40% and 60% B data as input to DryLab I-PLUS.

fairly straightforward, however, with the help of band-area data. The second and third bands appear to have changed positions between these two runs, and this might be difficult to recognize if these two bands had similar areas. The simulated run in Fig. 12C shows that for 50% B as mobile phase, bands 2 and 3 overlap to form a single peak. Comparison of this predicted separation with the actual run with 50% B (Fig. 12D) confirms that this band reversal has indeed taken place. Alternatively, with experimental data for three different mobile phases (Fig. 12A, B and D), it is relatively easy to match bands among these different runs.

Peak matching becomes more difficult when the organic solvent composition changes between two runs, as in Fig. 7. Changes in band position are less regular and predictable, and band areas occasionally change owing to changes in absorptivity. Problems of this type are even more pronounced when the mobile phase pH is varied, as the absorptivity of acids and bases at a given wavelength is often variable with pH, and changes in peak order can occur for changes in pH of only 0.1–0.2 units. The use of a diode-array detector (*e.g.*, refs. 21 and 22) is a partial solution to the problem of peak tracking, but this approach introduces considerably additional complexity.

7. CONCLUSIONS

Computer simulation as described here (using DryLab software) appears to represent an efficient, practical technique for facilitating HPLC method development. Most samples require only a few experimental runs (typically 4–8) plus about 1 h of computer time to achieve a successful separation. This approach to computer-assisted method development is strongly interactive, in that the chromatographer chooses an appropriate overall strategy. This contrasts with the "black box" approach used by optimization procedures (where the computer, rather than the user, is in charge) and allows the individual skills and insights of the user to contribute in solving the separation problem. Computer simulation is also flexible; for example, a previously developed method can be systematically improved with only one or two additional runs.

The present approach to computer simulation makes extensive use of "solvent-strength optimization", based on changes in band spacing as a result of changes in the mobile-phase composition (%B). A comparison of computer simulation with multi-solvent mapping procedures ("simplex lattice" and "sequential simplex") is shown here for two typical samples. In each instance, computer simulation based on solvent-strength optimization gave equivalent or better separations, despite the need for fewer experimental runs. This will not always prove to be the case, but it does suggest that computer simulation will often be a preferred approach for samples containing fewer than about a dozen components. An added advantage of computer simulation is its ability to use changes in column conditions to improve the separation further, after retention optimization is completed.

The same software (DryLab) used for solvent strength optimization can also be used for mapping separation as a function of other separation conditions, *e.g.*, pH, ternary solvent mixtures, temperature. The use of studies in which one parameter at a time is varied is less efficient than statistical-design strategies, and global optimima can be overlooked in this approach. However, we feel that this is often not an important consideration. Keeping the chromatographer "involved" is generally of greater value as method development proceeds.

Peak tracking and peak identification were discussed briefly. Neither of these requirements appears to pose a serious problem in computer simulation. One or two additional runs will usually suffice to confirm the identities of compounds in each band. Unknown samples (no standards available) present no special problems.

8. SUMMARY

Computer simulation (DryLab software) as a means of facilitating the development of isocratic high-performance liquid chromatographic methods is reviewed. The various features of computer simulation are discussed and several examples of its application are presented.

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