Journal of Chromatography, 485 (1989) 91–112
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 880

REVIEW

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

II. GRADIENT ELUTION

J. W. DOLAN, D. C. LOMMEN and L. R. SNYDER*
LC Resources Inc., 3 182C Old Tunnel Road, Lafayette, CA 94549 (U.S.A.)

CONTENTS

1. INTRODUCTION

A general discussion of computer simulation for use in high-performance liquid chromatographic (HPLC) method development was presented in Part I[1], with the aim of obtaining a final isocratic method. In this paper, the review is extended to the case of gradient elution. A number of examples of computer simulation for the development of gradient separations are shown as illustrations of various aspects of the DryLab® G software. A discussion of the accuracy of these simulations is also offered, together with recommendations for obtaining reliable predictions.

2. THEORY

In Part I[1] we discussed the basis of computer simulation as a means of developing a final isocratic HPLC method. The DryLab G software described in this paper

0021-9673/89/$03.50

also uses two experimental gradient runs as a means of determining the solute parameters k_w and S for each component of the sample (see the Discussion in Part I[1]). These parameters in turn determine the isocratic elution of each band as a function of the percentage of component B in the mobile phase (%B). In a similar fashion, once values of k_w and S have been measured, well established theory[2–5] allows us to predict a gradient separation for any combination of gradient conditions: gradient time (or steepness), initial and final values of %B and/or gradient shape. The effect of column conditions on separation (column length, particle size, flow-rate) can also be accounted for in the same way[1] as for isocratic elution. These relationships are in turn the basis of the present computer-simulation software (DryLab G)[6–10].

2.1. Practical aspects

Some other consequences of the theory of gradient elution should be noted. For linear gradients, we can define an average retention $\bar{k}$ for each band[a], given by

$$\bar{k} = 0.85 t_G F / V_m \Delta\Phi S \tag{1}$$

where t_G is gradient time, F is flow-rate, V_m is column dead volume, $\Delta\Phi$ is change in Φ during the gradient, Φ is the volume fraction of solvent B in the mobile phase and $S = \mathrm{d}(\log k')/\mathrm{d}\Phi$ (see Part I[1]). If this average retention $\bar{k}$ is held fixed, then there will be no change in band spacing as gradient or column conditions are varied. Hence, if we want to maintain constant band spacing (after optimizing the gradient and mobile phase conditions for the best band spacing and resolution), then $\bar{k}$ must remain constant. This will be the case, as long as the quantity $t_G F / V_m \Delta\Phi$ does not change.

Often we will keep the column dimensions and flow-rate the same while changing the gradient conditions. In this case, constant $\bar{k}$ requires constant $(t_G/\Delta\Phi)$, or a fixed gradient steepness (constant %B/min, with the column and flow-rate unchanged). If our gradient range has been optimized (for optimum band spacing and run time), and we want to vary the column plate number (column conditions) to improve the separation further, then it is necessary to hold $t_G F / V_m$ constant. That is, if we change the column length or flow-rate for a change in N, it is necessary to change t_G also. DryLab G adjusts for these effects automatically, as illustrated in following examples. For a further discussion of gradient-elution method development, see ref. 11.

3. EXPERIMENTAL AND SOFTWARE

These were described in Part I[1].

4. REPRESENTATIVE EXAMPLES OF METHOD DEVELOPMENT BASED ON COMPUTER SIMULATION

Method development based on DryLab simulations proceeds in essentially the same way, regardless of whether the final goal is an isocratic or a gradient method.

[a] $\bar{k}$ can be regarded as an average or effective value of k' for a solute band during gradient elution. It is equal to the instantaneous value of k' for each band when that band reaches the column mid-point (*i.e.*, when it has migrated half way through the column).

Therefore, it is suggested that the reader reviews one or more applications in Part I[1] as a background for the following discussion. The examples below were chosen to illustrate various features of computer simulation as an aid in developing a final gradient method.

4.1. Mixture of 16 polyaromatic hydrocarbons (PAHs)

The development of a gradient elution method based on computer simulation begins with two experimental runs where only the gradient time is varied, just as described in Part I[1] for the development of a final isocratic procedure. The present sample was therefore first separated using two 5–100% acetonitrile–water gradients in 20 and 60 min; Fig. 1 summarizes these results. Sixteen bands are recognizable in the 20-min gradient (A) and 15 bands in the 60-min run (B).

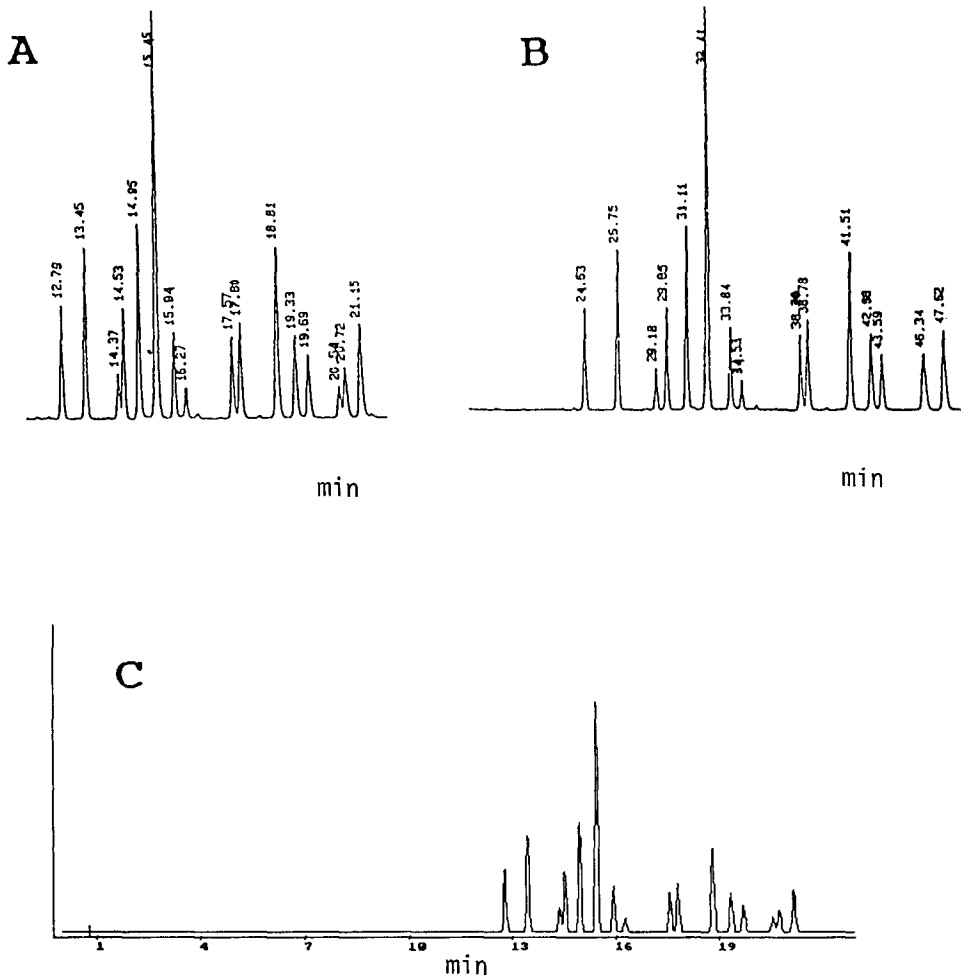

Fig. 1. Separation of a sixteen-component polyaromatic hydrocarbon sample. Column, 15 × 0.46 cm I.D., Supelcosil LC-PAH; 5–100% acetonitrile–water gradients; temperature, 35°C; flow-rate, 2 ml/min. See ref. 6 for details. (A) 20-min gradient; (B) 60-min gradient; (C) simulation of 20-min gradient.

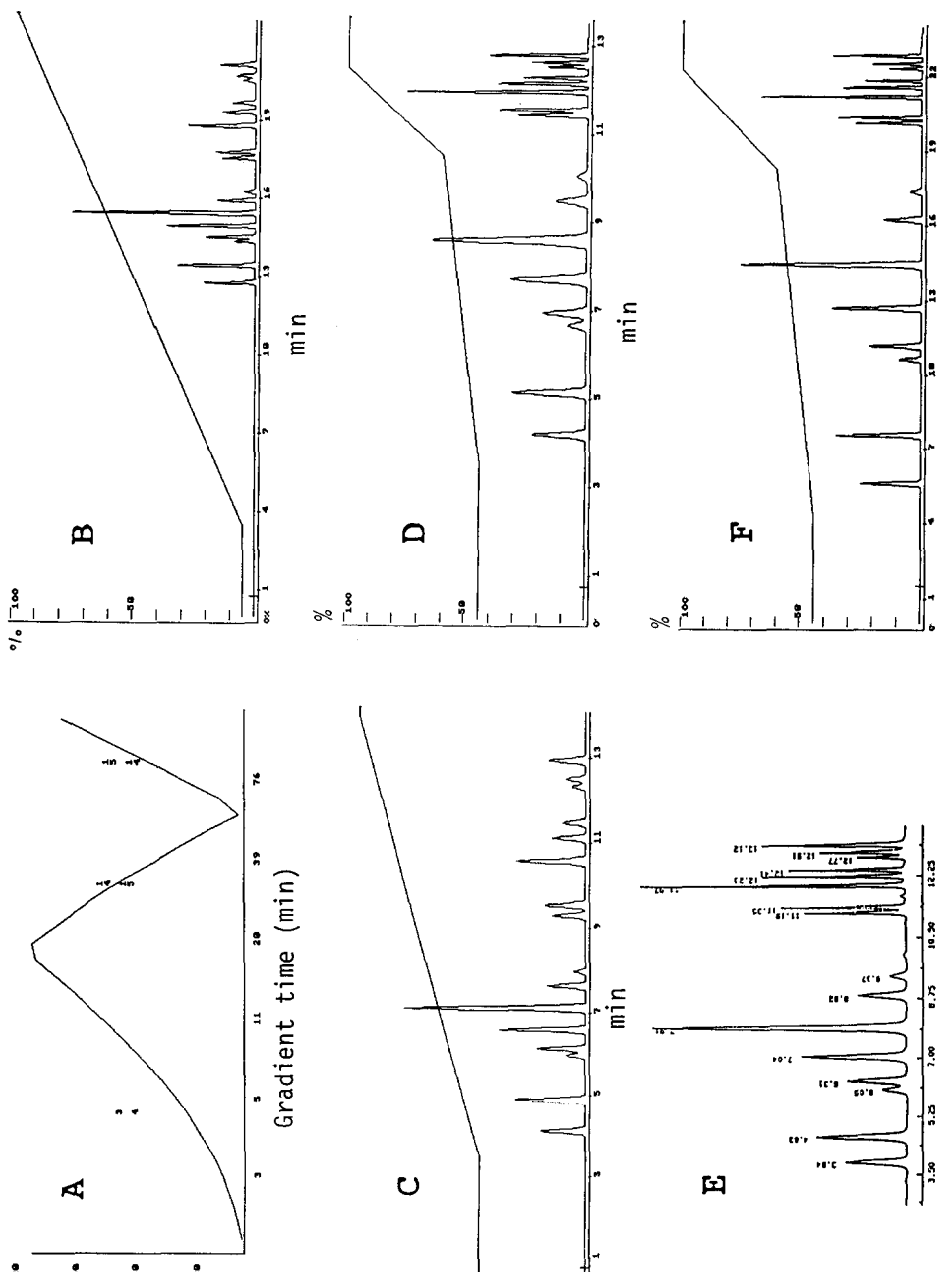

Fig. 2. Separation of a sixteen-component polyaromatic hydrocarbon sample. Conditions as in Fig. 1, unless indicated otherwise. (A) Resolution map (N = 6700) (based on 5–100% B gradient); (B) simulation for 5–100% B gradient in 19 min; (C) simulation for 44–93% B gradient in 10 min; (D) simulation for two-segment gradient (44–60–100% B in 0–7–9 min); (E) experimental run corresponding to conditions in D; (F) simulation for two-segment gradient as in D, except column length is 30 cm (44–60–100% B in 0–14–18 min).

These results were entered into the computer (same format as in Fig. 2 in ref. 1) to allow computer simulation to begin. Usually the best initial choice is to adjust the column plate number to match the experimental run (by entering an R_s value for a band pair from one of the initial experimental runs). This was done in the present example (run in Fig. 1A, $N = 6700$), which gave the simulated chromatogram in Fig. 1C. The latter is seen to closely resemble the experimental run in Fig. 1A.

The next step is to request a relative resolution map ($N = 6700$), as shown in Fig. 2A. The latter indicates that a resolution of $R_s \approx 1.0$ is possible for a gradient time of 19 min. The simulated chromatogram for these conditions is shown in Fig. 2B. Superimposed on Fig. 2B is the gradient we have chosen (5–100% B, linear shape) measured at the column outlet (notice the initial 4-min offset due to the equipment and column dead volumes). Visualization of the outlet gradient allows a better correlation of separation with changes in the gradient, and facilitates considerably the design of an optimum (or adequate) gradient. The numbers next to the plot in Fig. 2A correspond to critical band pairs, *i.e.*, those which are closest together (least resolved) for a given gradient time.

Returning to Fig. 2A, it is seen that the band spacing for bands 3–4 and 14–15 is very dependent on the gradient time (steepness). As a result, the resolution is highly sensitive to the choice of gradient time in this example. Similar behavior for other samples is fairly common[6–10,12,13], and is related to the changes in band spacing that occur in isocratic separation for a change in solvent strength (%B); see the discussion in Ref. 1.

The chromatogram in Fig. 2B shows that the initial bands elute at about 50% B, suggesting that the gradient can start at a %B value greater than the initial 5% B. Likewise, the last bands elute at about 90% B, suggesting that the final %B can be $< 100\%$. Shortening the gradient range in this manner can lead to a shorter run time, with no loss in resolution. Returning to Fig. 2A, it is seen that resolution is a strong function of gradient time or steepness. This means that if we shorten the gradient range, we must shorten the gradient time proportionately, so as to keep the gradient steepness constant (see discussion of eqn. 1). The gradient steepness in the separation in Fig. 2B is $(100 - 5)/19 = 5\%$ min. Hence if the gradient range is shortened, the gradient time must be reduced also to maintain a 5%/min gradient, otherwise the optimum band spacing will not be maintained and the resolution will decrease as suggested by the map in Fig. 2A.

With computer simulation we have the option of designing our next separation (with a reduced gradient range) on the basis of the above comments. That is, we might select a gradient range of 50–95% B in a time of $[(95 - 50)/5] = 9$ min (or any other values of gradient range and time). Alternatively, the DryLab G software will provide a recommendation for an optimum set of conditions (gradient range and time). In this case, the recommendation is for a gradient of 44–93% B in a time of 10 min. The simulated chromatogram for these conditions is shown in Fig. 2C. At this point we have a separation which is close to acceptable.

Our next step could be either (a) a change in column conditions for increased N and resolution or (b) further variation of the gradient conditions. The resolution map (Fig. 2A) is helpful in this regard; there we see that the resolution of the first critical band pair (3–4) increases with gradient time, whereas the resolution of the second critical band pair (14–15) decreases with gradient time. This suggests that a shallower

gradient for the separation of bands 3–4 followed by a steeper gradient for the separation of the remainder of the sample (including bands 14–15) should give a better overall resolution of the sample, and possibly also save time. Such a separation is shown in Fig. 2D (44–60–100% B in 0–7–9 min), with a resolution of R_s = 1.2; this is a significant increase in resolution (R_s = 1.0 in the separation in Fig. 2C) while maintaining a similar run time. The experimental run for the same conditions as in Fig. 2D is shown in Fig. 2E; this chromatogram agrees well with the predicted chromatogram in Fig. 2D.

Since the resolution in Fig. 2D and E is still marginal (R_s = 1.2), a further improvement in the separation can be obtained by increasing the column length or decreasing the flow-rate. Fig. 2F shows the predicted chromatogram (R_s = 1.7, 44–60–100% B in 0–14–18 min) for a 30-cm column *vs.* the 15-cm column in Fig. 2D and E. This run is acceptable in all respects; it is seen to have required a total of three experimental runs and about 1 h of computer time. Use of the usual trial-and-error experimental approach would undoubtedly have taken much longer.

4.1.1. Design of multi-segmented gradients. The preceding example illustrates the potential advantage of multi-segment gradients for the separation of moderately complex samples. Additional examples will be provided in following illustrations. Multi-segment gradients have often been used in the past, but usually for compressing parts of the chromatogram where there are only a few, widely separated bands, in order to save time. This is illustrated in Fig. 3 for the separation of a fifteen-component herbicide sample (data from refs. 6 and 7). In Fig. 3A, a linear 15–80% B gradient (in 18 min) is shown, with acceptable resolution (R_s 1.5) for all bands. As is commonly observed for samples containing ten or more bands, bands at the front (i) and back (iii) of the chromatogram are more spread out, whereas bands toward the middle (ii) tend to clump together with poorer overall resolution. This suggests the use of a three-segment gradient, where the initial and final segments are steeper in order to save run time.

A steeper segment at the end of the chromatogram is easily implemented, as shown in the example in Fig. 3B (15–45–80% B in 0–8.3–10 min; 8-min shorter run time *vs.* Fig. 3A). As the steepness of the initial segment (3.6%/min) is the same in Fig. 3A and B, bands eluted by this initial segment exhibit the same separation (R_s = 1.5) in each run. The final group of bands (iii) in Fig. 3B is still adequately resolved ($R_s > 1.5$), while the total gradient time has been reduced to 10 min *vs.* 18 min in Fig. 3A.

A further reduction in gradient time by using a three-segment gradient is shown in Fig. 3C (15–35–45–100% B in 0–2.0–4.7–6 min). The steepnes of the middle segment for group ii has been held constant, as in Fig. 3A and B, whereas earlier and later segments are steeper in order to shorten the overall run time. However, now the separation of group ii is seen to have changed markedly; two bands (indicated with an asterisk in Fig. 3C) overlap completely, so that R_s = 0 for the sample. This is a good illustration of potential problems in the design of mult-segmented gradients, at least when the band spacing changes with gradient steepness. In such cases, the steepness of an earlier gradient segment can affect the separation of bands eluted in a later segment. In practice, this means that the design of optimum multi-segment gradients may require a large number of trial-and-error experiments, although a number of useful rules can be offered in this regard[9,10]. In many instances, the design of opti-

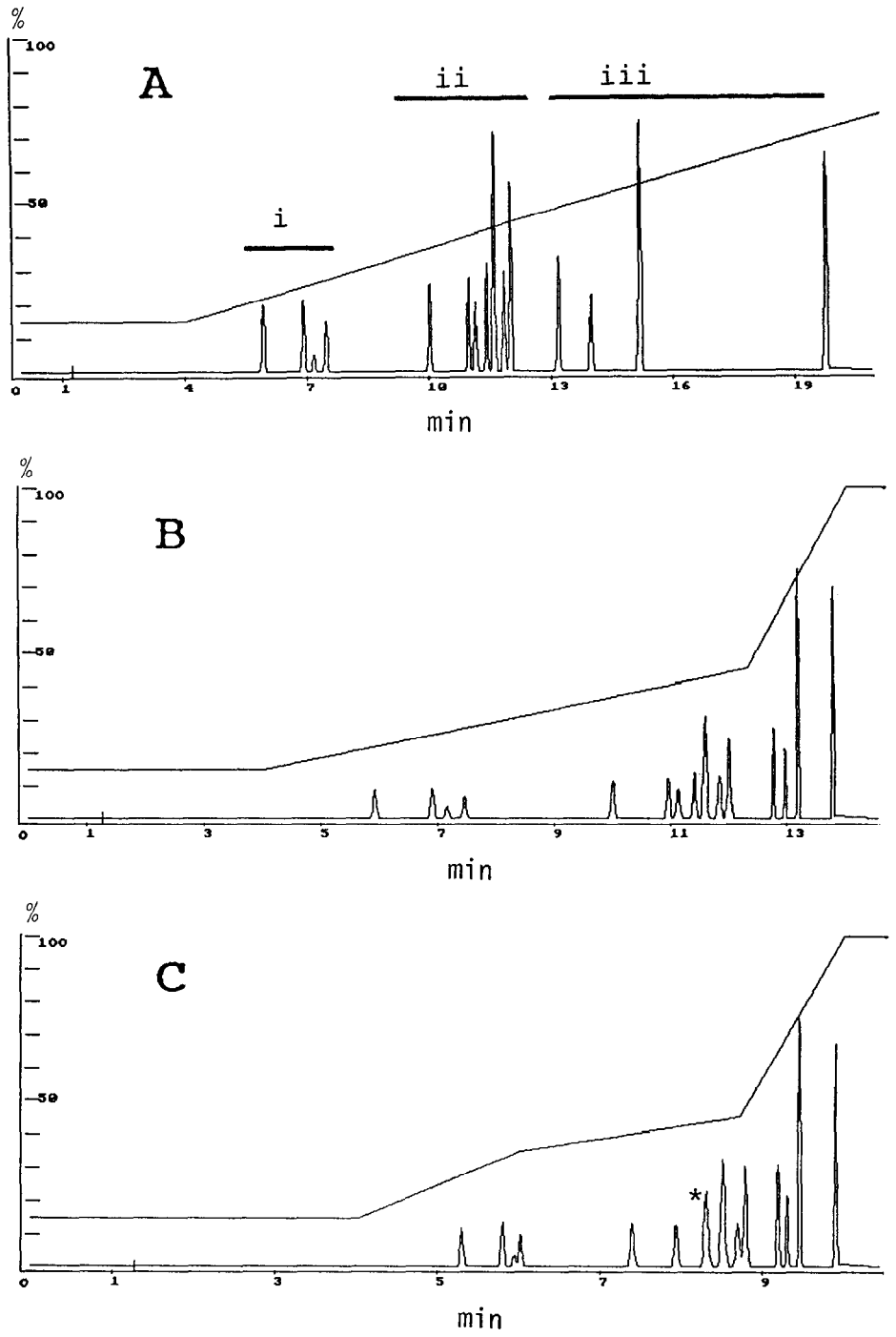

Fig. 3. Simulated separations of a fifteen-component herbicide sample, showing the effects of segmented gradients for shortening run time. Column, 25 × 0.46 cm I.D. Zorbax C_8 (DuPont); 15–100% B gradients; temperature 35°C; flow-rate 2 ml/min. See refs. 6 and 7 for details. (A) 15-80% B in 18 min; (B) two-segment gradient (15–45–80% B in 0–8.3–10 min); (C) three-segment gradients (15–35–45–100% B in 0–2.0–4.7–6.0 min).

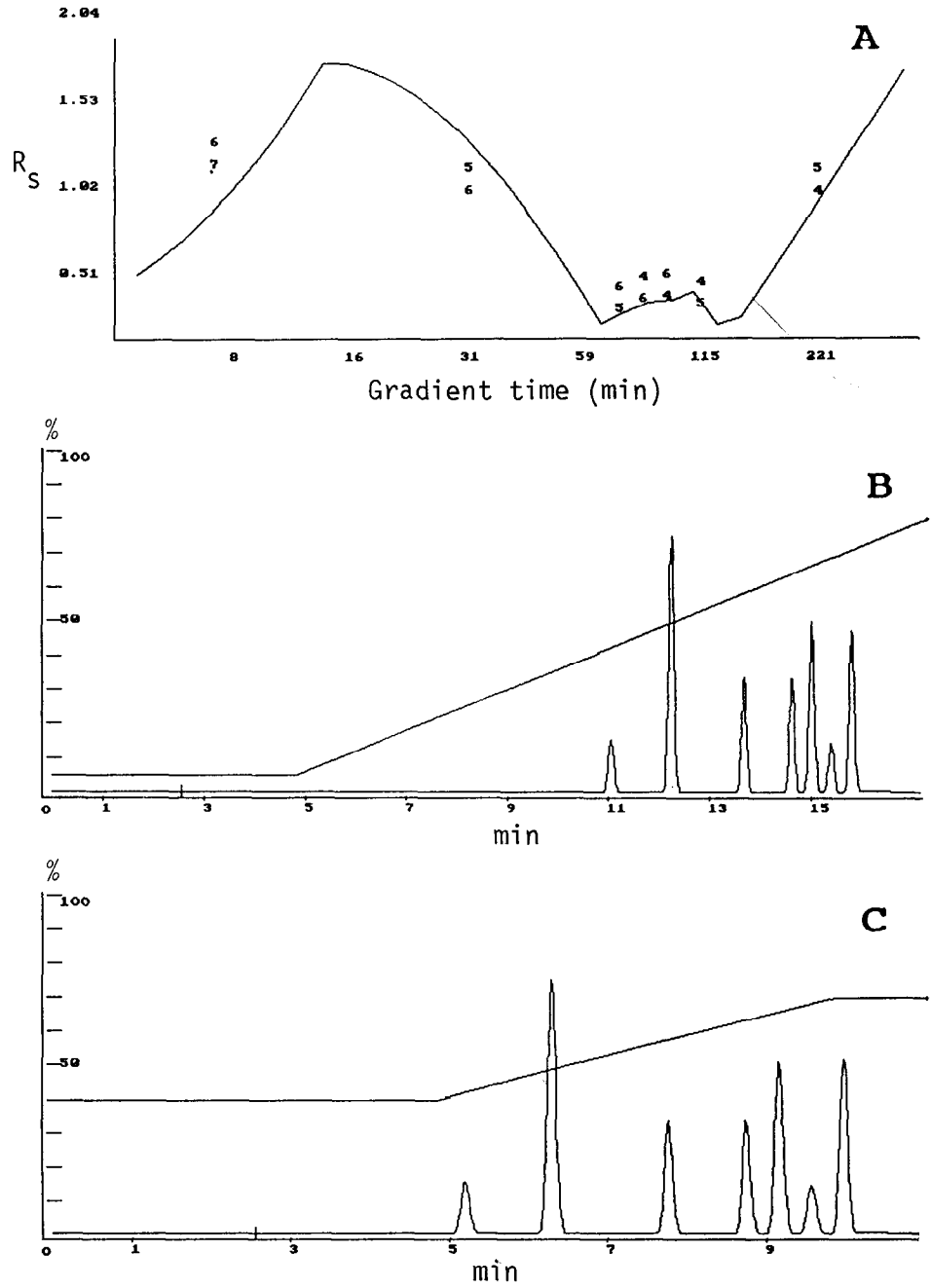

Fig. 4. Simulated (gradient elution) separations of a seven-component subsituted-benzene sample. (A) resolution map (based on 5–95% B gradient); (B) 5–95% B gradient in 16 min; (C) 40–70% B gradient in 5.3 min. See ref. 1 for details.

mized multi-segment gradients is only practical by using computer simulation (in place of actual runs in the laboratory).

4.2. Mixture of seven substituted benzenes

The separation of this sample was described in Part I[1]. It was observed there that the resolution changes markedly for different %B values (see Fig. 8A in ref. 1); acetonitrile–water (55:45, v/v) gave a good separation of the sample (R_s = 2.5). However, this was only possible for undesirably small values of k' (k' = 0.3) for the first band. In such cases, it is often possible to achieve good resolution and $1 < k' < 20$ for all bands by using gradient elution instead of isocratic separation. This is illustrated in Fig. 4. Using the same two experimental runs (Fig. 6 in ref. 1) as input data for the computer, the resolution map in Fig. 4A resulted. This map has the same general form as the map in Fig. 8A in ref. 1 (because band spacing changes in the same general way for increase in either gradient time or isocratic %B). From Fig. 4A, it is predicted that a 5–95% B gradient in 16 min will yield acceptable resolution of the sample (R_s = 1.7). The resulting simulated chromatogram is shown in Fig. 4B, confirming this expectation.

The chromatogram in Fig. 4B wastes time at the beginning and end of the run, suggesting the use of a narrower gradient range (but with the same steepness of 5.6%/min B). Fig. 4C shows such a separation (40–70% B in 5.3 min, R_s = 2.0), which is a good final choice. This is a better alternative than the isocratic method in Part I[1].

4.3. Mixture of 23 peptides

A sample containing 23 synthetic peptides with molecular weights between 500 and 4000 Da was prepared as a means of evaluating the present and other[14] software. Method development was begun by carrying out two gradient separations with the conditions shown for Fig. 5 A and B [gradient times of 45 min (a) and 180 min (b)]. A total of 22 bands can be seen in each chromatogram[a] (excluding minor non-peptide peaks), meaning that there is a band overlap in each run. The use of band areas and relative retentions allows the various peaks to be matched between these two runs, including the identification of overlapping bands.

The run conditions, retention times and band areas from Fig. 5A and B were next entered and computer simulation was begun. The column plate number was first adjusted so as to match the resolution between simulated and experimental runs. The resulting simulation for the 45-min gradient (Fig. 5A) is shown in Fig. 5C (N = 10 000). At this point,, a relative resolution map was requested (Fig. 5D). The resolution is seen to vary markedly with gradient time, even for small changes in this variable, *i.e.*, the band spacing changes markedly with gradient time for this sample. For this reason, the resolution is critically dependent on a particular gradient time. From Fig. 5D we also see that a gradient time of about 85 min provides maximum resolution, R_s = 1.1.

The selection of a linear gradient from 5 to 50% in 85 min represents a reasonable choice of separation conditions for this sample. The predicted separation is

[a] Fig. 5B appears to contain fewer than 22 bands, but this is due to the poor resolution of some bands (these bands and their retention times were picked up by the data system).

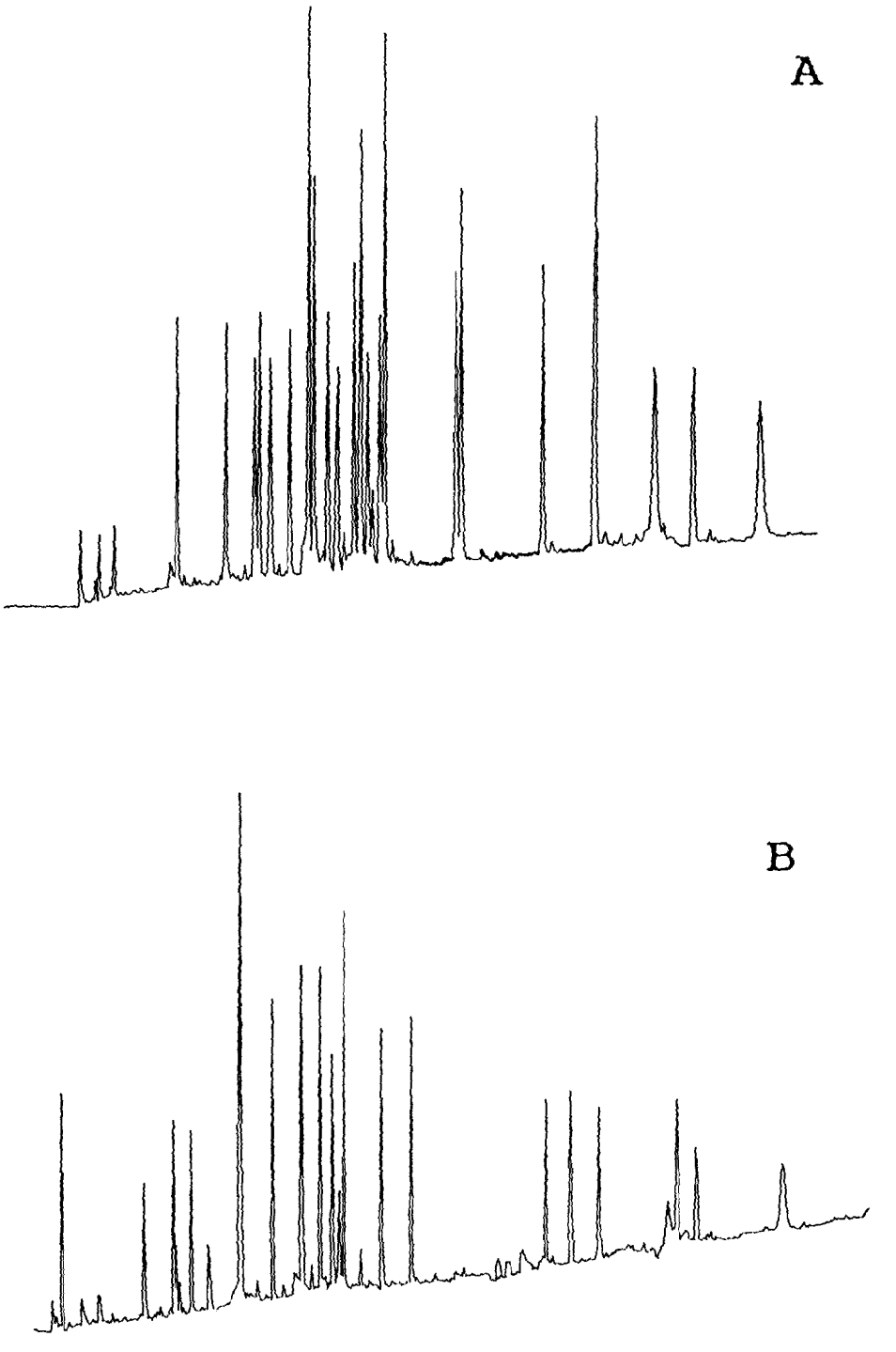

Fig. 5.

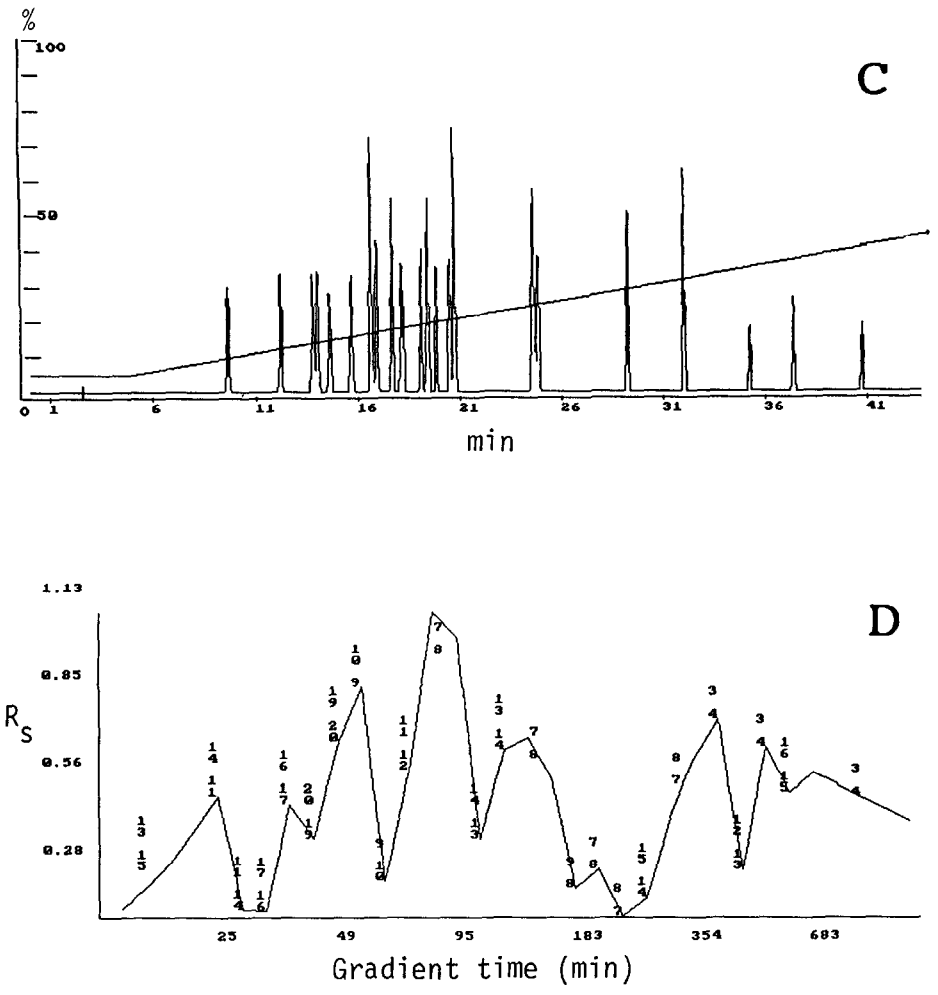

Fig. 5. Separation of a sample containing 23 synthetic peptides. Column, 25 × 0.46 cm I.D. Zorbax Rx (DuPont); 5–50% B gradients at 1.0 ml/min (0.1% TFA–water–acetonitrile); temperature, 30°C. See ref. 1 for details. (A) Experimental 45-min run; (B) experimental 180-min run; (C) simulated 45-min run [same conditions as in (A); N = 10 000]; (D) relative resolution map (based on 5–50% B gradient).

shown in Fig. 6A. In this instance, two experimental runs (Fig. 5A and B) plus about 10 min of computer time provided a good start to a method for this sample. However, it is possible to improve considerably on this choice by further computer simulations. For the present sample, it was found (computer simulation) that a three-segment gradient (5–21–26–50%B in 0–29–33–45 min) gives a resolution R_s = 1.1 in only 45 min (Fig. 6B); *i.e.*, half the time for a linear gradient in 85 min.

DryLab G also allows the user to change the column dimensions, particle size or flow-rate so as to vary the column plate number. This option was used to optimize this separation further; for a flow-rate of 0.4 ml/min (instead of 1 ml/min originally), it was predicted that the resolution could be increased to R_s = 1.4 in a total time of

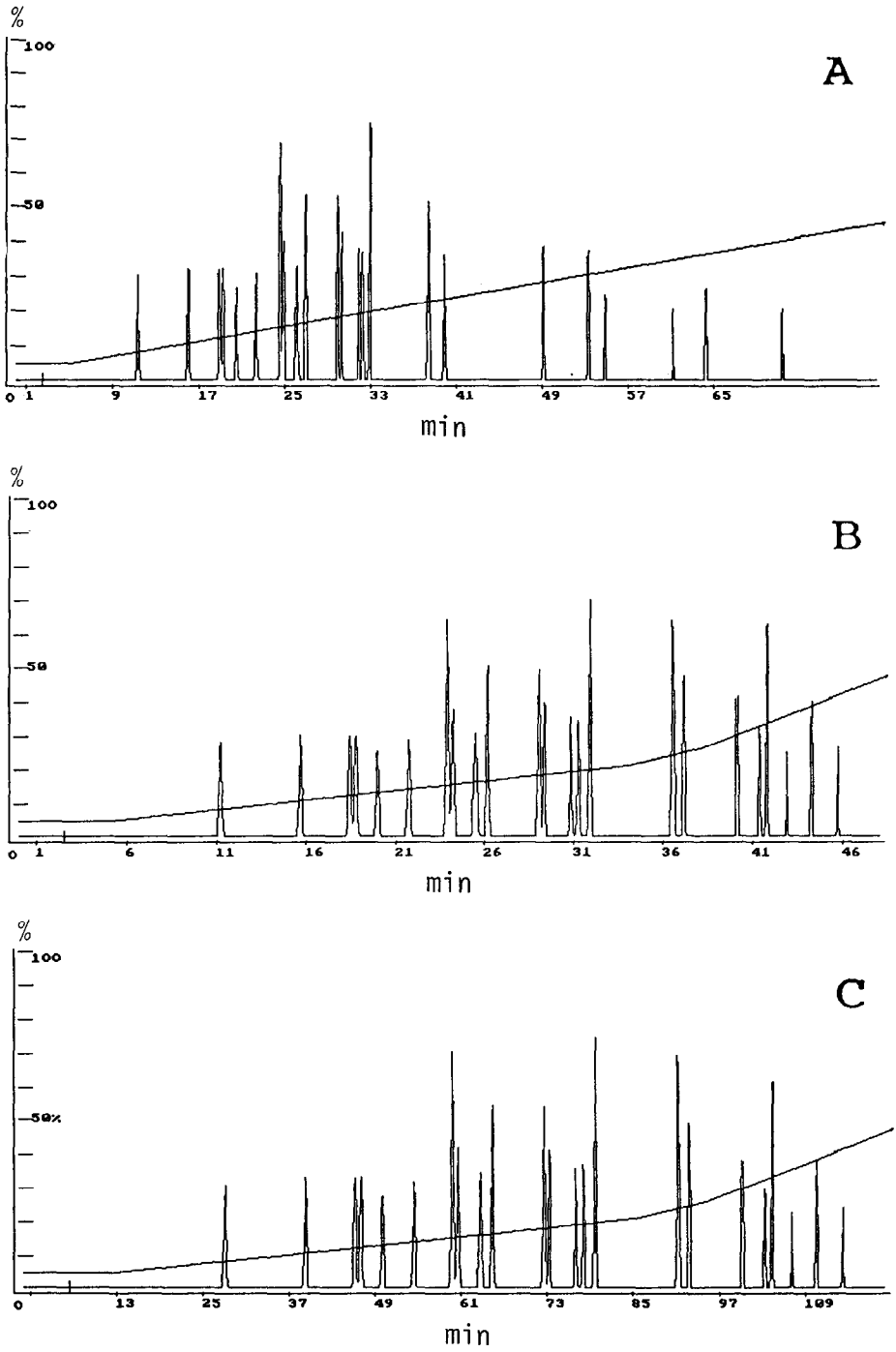
%
100
50
A
0 1 9 17 25 33 41 49 57 65
min
%
100
50
B
0 1 6 11 16 21 26 31 36 41 46
min
%
100
50%
C
0 13 25 37 49 61 73 85 97 109
min

Fig. 6.

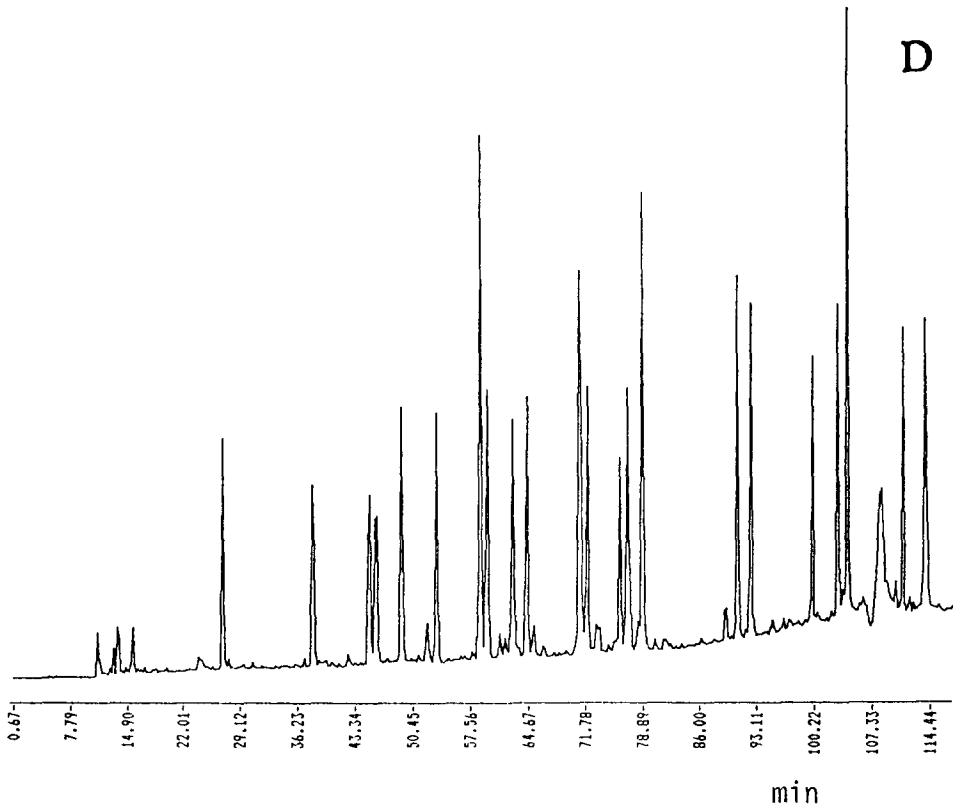

Fig. 6. Optimized separations of synthetic peptide sample in Fig. 5; conditions the same unless indicated otherwise. (A) Simulated separation for optimum gradient time of 85 min (linear 5–50% B gradient); (B) simulated separation for multi-segment gradient (5–21–26–50% in 0–29–33–45 min); (C) simulated separation for gradient in (B), except flow-rate 0.4 ml/min (0–5–21–26–50% in 0–72.5–82.5–112.5 min); (D) experimental run for conditions in (C).

112 min[a]. The simulated chromatogram for these conditions is shown in Fig. 6C and the experimental chromatogram is compared with it in Fig. 6D. The comparison between the predicted and actual chromatograms is fairly good. Retention time predictions agree with the experimental values within ± 1.4% (C.V.), and resolution is predicted within ± 8% (C.V.).

4.4. Ribosomal proteins from E. coli

The ribosomal proteins consist of 53 proteins which can be separated by zonal centrifugation into two fractions: 30S (21 proteins) and 50S (32 proteins) proteins. Although several groups have attempted the separation of these fractions by re-

[a] Following the optimization of gradient conditions (shape, time, initial and final %B), the band spacing can be maintained constant (while the column conditions are varied) by changing the gradient time. In the present example, the flow-rate was reduced from 1.0 to 0.4 ml/min, which required an increase in the overall gradient time by a factor of 1.0/0.4 = 2.5.

versed-phase HPLC, until recently no-one has reported the complete separation of either sample in a single run (the best previous separations leave several band pairs unresolved). The use of computer simulation[8,10] has changed this situation dramatically, permitting the separation in a single reversed-phase run of all 21 30S proteins and 31 of the 32 50S proteins. Here we summarize the application of computer simulation to the separation of the 50S ribosomal proteins.

Fig. 7 shows the initial two experimental runs for the 50S proteins, with gradient times of 192 and 720 min. Detection at both 214 and 280 nm was used as an aid in matching bands between the two runs. The column plate number was adjusted to

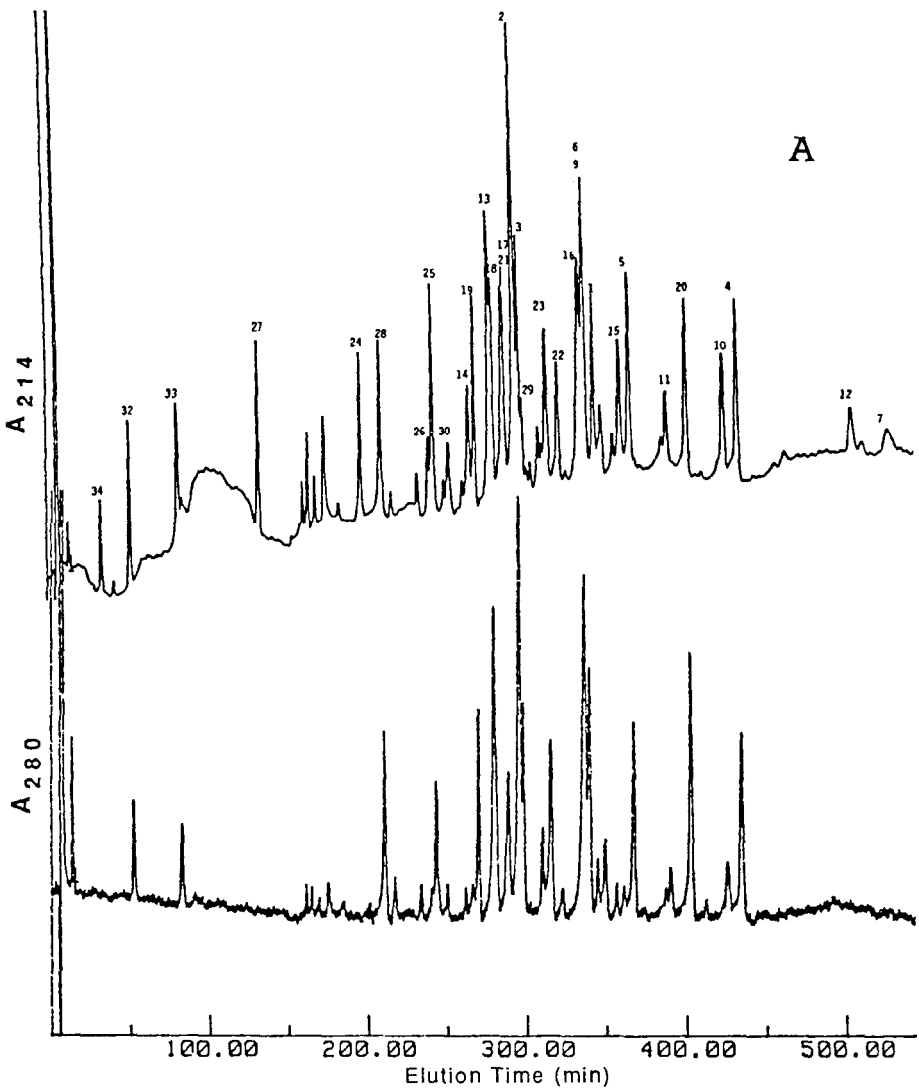

Fig. 7.

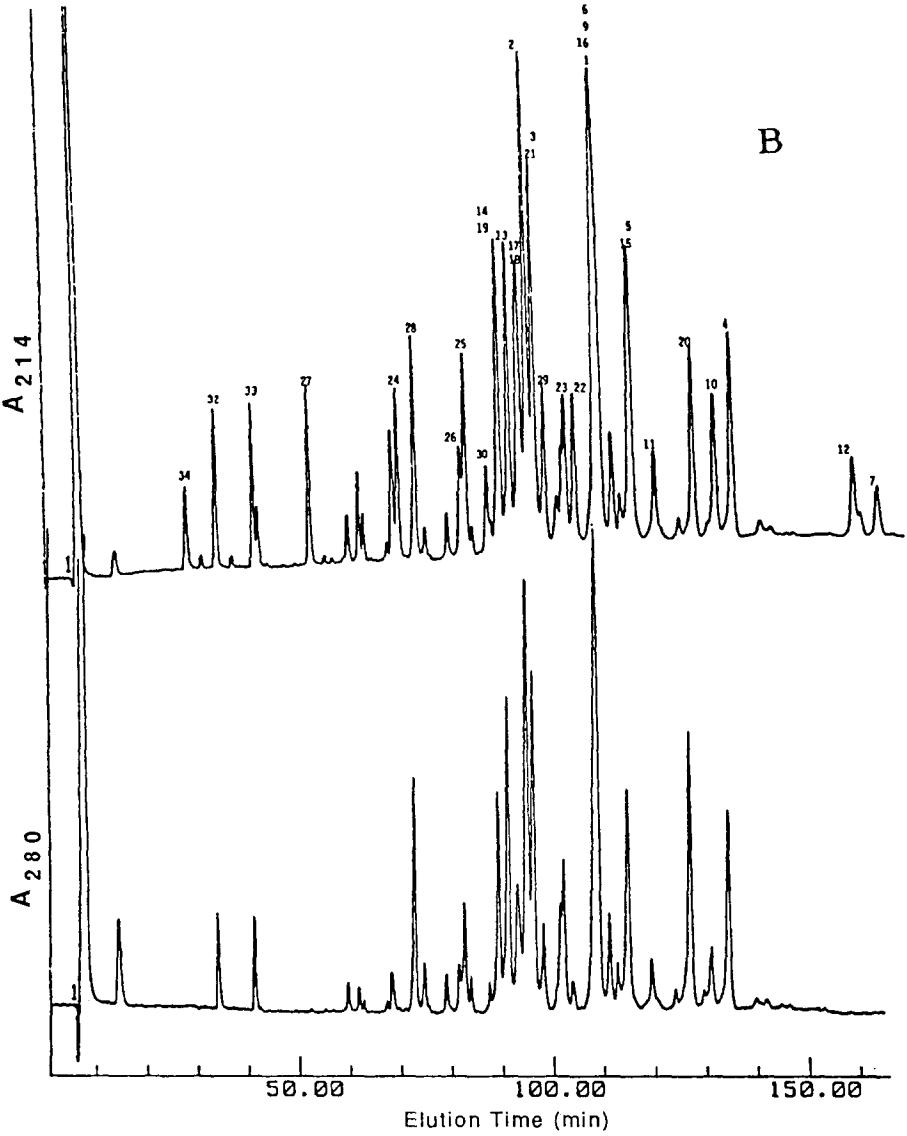

Fig. 7. Separation of 50S ribosomal proteins. Column, 25 × 0.46 cm I.D. Zorbax Protein PLUS (DuPont); 18–66% B (acetonitrile–water gradient with added triethylamine plus TFA); flow-rate 0.7 ml/min at 25°C; see ref. 15 for details. (A) Gradient time of 192 min; (B) gradient time of 720 min. Detection (A and B): top, 214 nm; bottom, 280 nm.

N = 900, in order to match the resolution of experimental and predicted runs, and a relative resolution map was requested (Fig. 8A). This suggests that the maximum possible resolution is R_s = 0.8[a] for an 8-h run.

[a] One band pair was unseparated with any choice of gradient time; therefore, data for 31 different bands were entered into the program for computer simulation and values of R_s were calculated on the basis of 31 different sample components, assuming a linear gradient from 18 to 66% B.

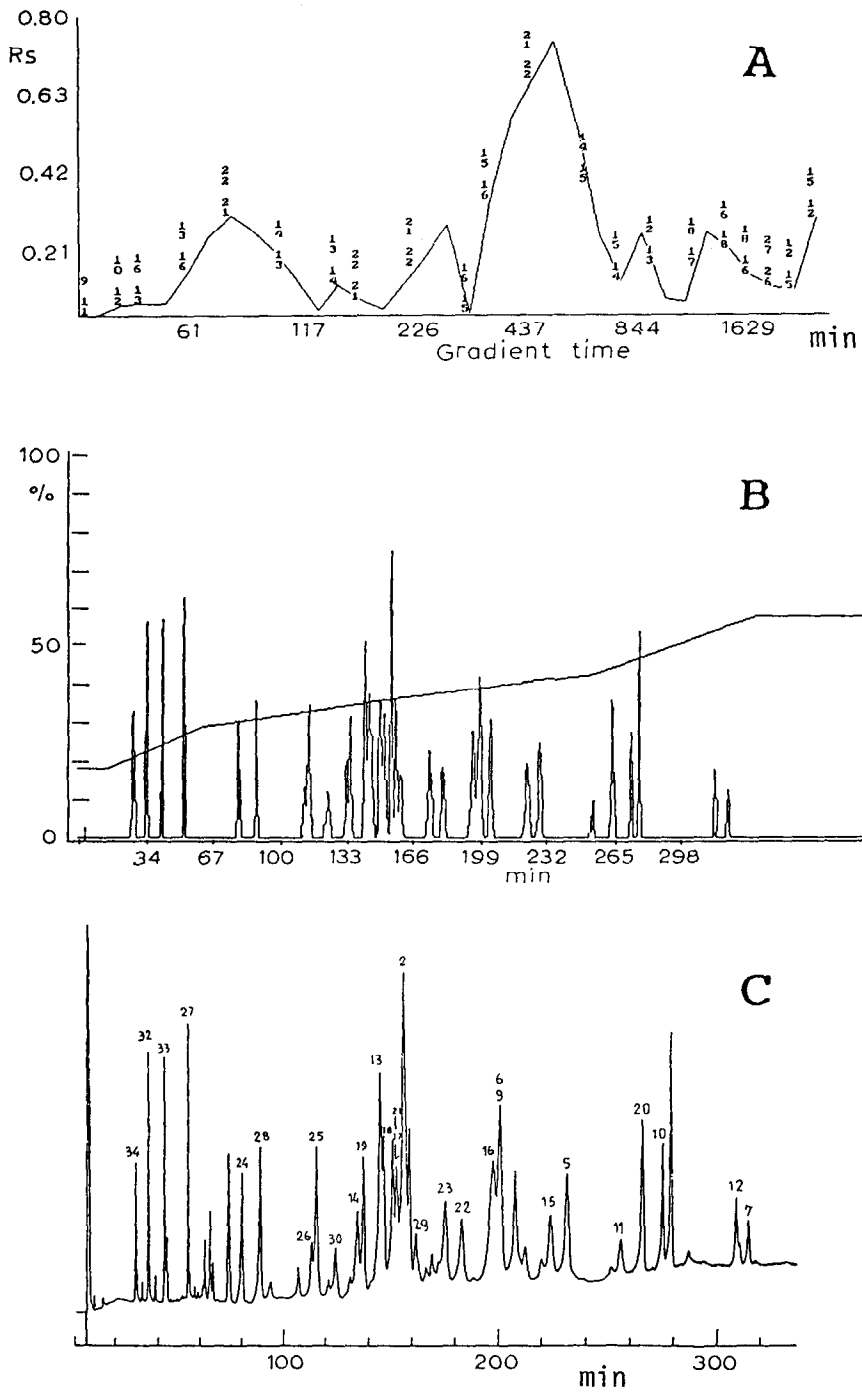

Fig. 8. Separation of 50S ribosomal proteins. Conditions as in Fig. 7, unless indicated otherwise. (A) Relative resolution map (N = 900) (based on 18–66% B gradient); (B) predicted optimum separation (18–29–37–43–58% B in 0–46–142–241–320 min); (C) experimental run corresponding to that in (B).

The use of a multi-segmented gradient was found to improve this separation, with $R_s = 0.9$ possible in 5 h. This predicted separation is shown in Fig. 8B. Finally, the experimental run corresponding to the conditions in Fig. 8B is shown in Fig. 8C. Agreement between the two chromatograms is good: retention times, ±0.5% (S.D.); resolution, ±8% (S.D.).

5. ERRORS IN COMPUTER SIMULATION AND HOW TO AVOID THEM

By now the use of computer simulation as an aid in HPLC method development should be clear. Put simply, computer simulation allows the user to carry out equivalent trial-and-error separations with much less effort and in much less time than in corresponding experimental runs. The major question then is how well the computer predictions match reality. The answer is that computer simulations can be fairly accurate, but this requires some attention to the experimental procedures and equipment. In addition, the initial experimental separations used for computer simulation must be chosen carefully; however, DryLab will warn the user when the experimental conditions selected are inappropriate for predicting a certain separation. Finally, errors in computer prediction can be caused by certain other factors. In this section, we provide a brief review of what is now known concerning computer-simulation errors and means for minimizing them. With a few exceptions, the discussion will apply to both isocratic and gradient predictions.

5.1. Comparisons of experimental vs. computer-simulation results

The comparisons offered here and in Part I[1] suggest that computer simulation can be adequately accurate for the purposes of method development. We have found[5,6,8–10,16–18] for a broad range of samples that retention times can generally be predicted with an accuracy of about ±1–3%, and resolution is predictable within ±5–10%. Provided that computer simulations are this good or better, there will be no problem in using simulated results for method development. Errors in predicted separations are usually greater when two gradient runs are used to predict an isocratic run, and smaller for the use of two isocratic runs to predict an isocratic run or two gradient runs to predict a gradient run. There is usually no reason for using isocratic data to predict gradient runs, and DryLab does not provide for this possibility.

5.2. Causes of error in computer simulation; means for reducing these errors

Some potential errors in computer simulation are listed in Table 1 (many of these effects are equally important for other computer-assisted method-development procedures). We shall discuss each of these problems in turn.

5.2.1. Equipment problems. For reliable computer-simulation studies, especially where experimental gradient runs are used as input, it is important that the *actual* gradient be know fairly accurately. The experimental gradient can differ from the gradient selected by the user for various reasons. For this reason, it is important to measure the gradient shape[18] with a blank run (no sample) and the column removed from the system[a], as illustrated in the example in Fig. 9A. Generally the experimental

[a] With the sample injector connected directly to the detector (column removed), use methanol as the solvent for each of the two solvent reservoirs. Add about 0.01% of acetone to the solvent A reservoir and 0.1% of acetone to the solvent B reservoir; adjust the detector response (wavelength, sensitivity setting) to obtain 90% of full-scale absorbance for solvent B. Then run a blank gradient from 0 to 100% B in 20 min, with a flow-rate of 1 ml/min.

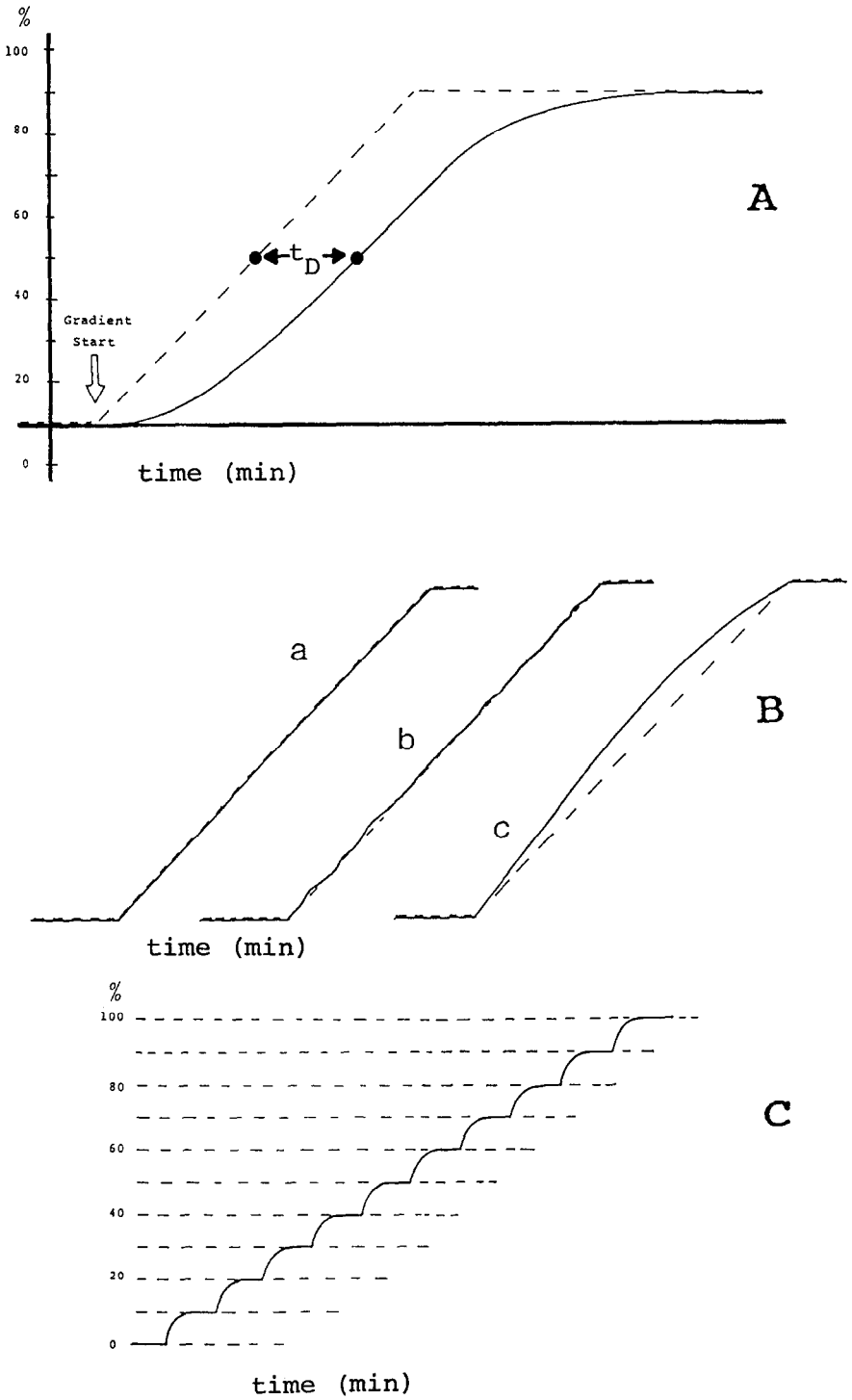

Fig. 9. Gradient shape as a function of the HPLC system. See text for details. (A) Typical blank gradient with column removed (solid line, experimental gradient; dashed line, expected gradient); (B) examples of equipment malfunction; (C) test of accurate proportioning by gradient system.

TABLE 1

POTENTIAL SOURCES OF ERROR IN COMPUTER SIMULATION

Factor	*Comment*
Equipment	Problems can arise from gradient or pumping errors, inaccurately measured dwell volumes or excessive rounding of the gradient due to dispersion effects
Column processes	Errors can be caused by demixing of the mobile phase within the column
Change in column	Loss of bonded phase or column deactivation during use can lead to changes in retention, in turn leading to errors in computer simulation
Errors in interpreting the chromatogram	Overlapping bands can cause errors in measured retention times; overlooked band reversals can lead to error in predicted retention times and separation
Non-linear plots of log k' *vs.* %B	Non-linear retention plots can lead to errors in predicted retention, particularly for extrapolated runs

gradient (solid curve in Fig. 9A) will be rounded at the ends and shifted by the dwell time t_D. Gradient rounding is generally not significant, provided that the errors at the gradient ends are less than 1–2%. The gradient dwell time must be corrected for, however, so it is important that this quantity be accurately known (as in Fig. 9A) for the HPLC system being used[a]. The dwell volume $V_D = t_D F$ (where F is flow-rate) is used in DryLab calculations whenever gradient data are involved.

Apart from delay and rounding of the gradient, it is not uncommon to find HPLC systems that exhibit errors in the way the gradient is formed. These are illustrated in Fig. 9B and C. In Fig. 9B, an error-free gradient is illustrated in (a). The corresponding gradient in (b) shows a "wobble" that is due to solvent misproportioning, while the gradient of (c) shows a consistent curvature for what was intended as a linear gradient. Gradients as in (b) can arise from inadequate degassing of the mobile phase, although equipment imperfections are also a possible cause. Curvature as in (c) can usually be corected by adjustment of the equipment. If inaccurate predictions are obtained from computer simulation, it is also useful to check the proportioning accuracy as illustrated in Fig. 9C. See the further discussion in refs. 18–20.

5.2.2. Column processes. The main problem of this type is demixing of the mobile phase during the gradient. The strong solvent B will be sorbed to some extent by the stationary phase, causing a small delay of the gradient reaching the end of the column. This effect has been studied in detail[18] for reversed-phase HPLC, and in most instances mobile-phase demixing will not cause significant errors in predictions by computer simulation. Predicted retention times may be slightly too small, but the resolution will generally not be affected. The combination of small, narrow-pore columns ($V_m < 1.0$ ml, pore diameter < 10 nm) and wide-range gradients (*e.g.*, 0–100% B) leads to the largest errors in the gradient. Beginning the gradient at 5% B or higher also helps to avoid problems with both column equilibration and mobile-phase demixing.

[a] The dwell volume can also be determined by carrying out three experimental gradient runs (for the sample being studied) by computer simulation, *e.g.*, with gradient times of 20, 40 and 60 min. Data for two runs (*e.g.*, 20 and 40 min) can be entered into DryLab G, and the 60-min run can be predicted. The dwell volume used for computer simulation can then be adjusted so as to give good agreement between predicted and actual 60-min runs; the best fit will be given by the correct dwell volume.

5.2.3. Change in the column. Continued use of an HPLC column causes its gradual deterioration, including loss of bonded phase (especially at high or low pH) and build-up of strongly held sample components on the column[8,20]. Each of the latter processes affects sample retention and the ability to obtain the same separation (same separation conditions) at different times. This problem can have serious effects for any method-development procedure, but it is less of a problem for computer simulation, because fewer experimental runs are required during method development with less overall change in column retention.

Reproducibility of retention should be confirmed from time to time with the column being used for method development (by running the sample under some set of standard conditions). We have also found (see the discussion in ref. 8) that changes in column retention can be minimized by (a) conditioning a new column before use, (b) carrying out all computer simulation runs within a short time interval (1–2 days) and (c) dedicating a specific column to runs required for a particular method development study.

5.2.4. Errors in interpreting the chromatogram. These are of two kinds: (a) inaccurate retention times and (b) misassigned bands. Inaccurate retention times arise mainly from overlapping bands, where band maxima are shifted due to overlap[8]. This problem is generally not serious in computer simulation, except for the case of very complex samples that exhibit extensive changes in band spacing as conditions are varied. When errors of this type are discovered, it is possible to adjust the initial (experimental) retention time data to correct for this problem; see the further discussion in ref. 8.

Errors due to misassigned bands (errors in peak tracking) generally lead to major errors in computer simulation. Such errors are usually obvious, however, when predicted separations are compared with experiment; see the discussion of peak tracking in Part I[1].

5.2.5. Non-linear plots of log k' vs. %B. DryLab predictions assume that log k' changes linearly with %B. In general, this is not precisely true, and sometimes the curvature of such plots can be noticeable. A detailed analysis of such effects for reversed-phase separation[5,17] leads to the conclusion that errors in computer simulation as a result of non-linear retention plots are generally not serious. It is necessary to choose appropriate conditions for the initial two experimental runs[5]: a difference in gradient times of 3–4-fold and a difference in %B values (isocratic runs) of at least 10% B.

Predictions based on these starting runs will be most accurate when run conditions are interpolated between experimental data, and less accurate as predictions are extrapolated a greater distance from the starting runs. DryLab monitors the choices of starting (experimental) conditions and of various simulations, and advises the user when an error in predicted results is likely. As a result, significant errors due to non-linear retention plots are unlikely for reversed-phase separations when either %B or the gradient conditions are varied.

The possibility of error is significantly greater when other variables are being mapped (pH, composition of organic solvent mixtures); see the discussion of Table II in Part I[1]. Likewise, the use of DryLab G for simulating separations by ion-exchange or normal-phase HPLC is also more prone to error. However, even in these cases the analysis of ref. 5 suggests that such errors will often not be so serious as to preclude

the use of DryLab computer simulation. The ion-exchange separation of proteins, for example, is predictable with about the same accuracy as reversed-phase separations owing to the larger z values involved (see Discussion in ref. 5 and examples in ref. 22).

6. CONCLUSIONS

Computer simulation can be used to reliably predict HPLC separations as a function of the gradient conditions: change in gradient time or steepness, change in initial and final mobile-phase composition (%B) and change in gradient shape (multi-segment gradients). This means that the development of a final gradient elution method can be considerably improved by using computer simulation to complement actual experimental runs. The time required is much less, and much better separations can usually be achieved by carrying out a large number of trial-and-error runs based on computer simulation. The accuracy of such predictions for both small and large molecules, and for samples of biological or non-biological origin, is generally adequate for the purposes of method development; *i.e.*, ± 1–3% in retention times and ± 5–10% in the resolution of individual band pairs.

It is now well established that band spacing changes significantly for most samples as the gradient steepness is varied. This means that the selection of an optimum gradient steepness is often critical for a good final separation by gradient elution. The choice of the best gradient steepness for a given sample can be arrived at within a few minutes by computer simulation, based on the availability of resolution maps as a function of gradient time. Alternative trial-and-error experimental runs can require several days in the laboratory.

In many instances, it is desirable to select an optimum gradient steepness for different parts of the chromatogram, favoring the use of multi-segment gradients. The design of a suitable overall gradient in this way can require a large number of runs to arrive at a good final separation. With computer simulation this is possible within 2–4 h for typical samples. It is unlikely that this powerful tool for optimizing gradient separations can be used with conventional trial-and-error experiments because of the long time required, changes in the column during use and other factors.

Computer simulations are subject to certain errors which have been reviewed in this paper. By attention to various experimental problems, these errors can be made insignificant in most instances. The DryLab software incorporates a number of internal checks that can detect predictable errors and advise the user of appropriate action.

7. SUMMARY

Computer simulation (DryLab software) as an aid for the development of gradient high-performance liquid chromatographic methods is reviewed. Several examples of its application are presented and the accuracy of such predictions is discussed.

REFERENCES

1 L. R. Snyder, J. W. Dolan and D. C. Lommen, *J. Chromatogr.*, 485 (1989) 65.
2 L. R. Snyder, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography —Advances and Perspectives*, Vol. 1, Academic Press, New York, 1980, p. 207.

3 P. Jandera and J. Churáček, *Gradient Elution in Column Liquid Chromatography—Theory and Practice*, Elsevier, Amsterdam, 1985.
4 M. A. Stadalius and L. R. Snyder, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography—Advances and Perspectives*, Vol. 4, Academic Press, New York, 1986, p. 195.
5 M. A. Quarry, R. L. Grob and L. R. Snyder, *Anal. Chem.*, 58 (1986) 907.
6 J. W. Dolan, L. R. Snyder and M. A. Quarry, *Chromatographia*, 24 (1987) 261.
7 J. W. Dolan and L. R. Snyder, *LC · GC, Mag. Liq. Gas Chromatogr.*, 5 (1987) 970.
8 B. F. D. Ghrist, B. S. Cooperman and L. R. Snyder, *J. Chromatogr.*, 459 (1989) 1.
9 B. F. D. Ghrist and L. R. Snyder, *J. Chromatogr.*, 459 (1989) 25.
10 B. F. D. Ghrist and L. R. Snyder, *J. Chromatogr.*, 459 (1989) 43.
11 L. R. Snyder, J. L. Glajch and J. J. Kirkland, *Practical HPLC Method Development*, Wiley-Interscience, New York, 1988, Ch. 7.
12 J. L. Glajch, M. A. Quarry, J. F. Vasta and L. R. Snyder, *Anal. Chem.*, 58 (1986) 280.
13 M. Kunitani, D. Johnson and L. R. Snyder, *J. Chromatogr.*, 371 (1986) 313.
14 C. T. Mant, T. W. L. Burke, N. E. Zhou, J. M. R. Parker and R. S. Hodges, *J. Chromatogr.*, 485 (1989) 365.
15 B. F. D. Ghrist, *Ph.D. Thesis*, University of Pennsylvania, Philadelphia, PA, 1989.
16 L. R. Snyder, M. A. Quarry and J. L. Glajch, *Chromatographia*, 24 (1987) 33.
17 L. R. Snyder and M. A. Quarry, *J. Liq. Chromatogr.*, 10 (1987) 1789.
18 M. A. Quarry, R. L. Grob and L. R. Snyder, *J. Chromatogr.*, 285 (1984) 1 and 19.
19 J. W. Dolan, *LC · GC, Mag. Liq. Gas Chromatogr.*, 7 (1989) 7.
20 J. W. Dolan and L. R. Snyder, *Troubleshooting HPLC Systems*, Humana Press, Clifton, NJ, 1989.
21 T. Sasagawa, Y. Sakamoto, T. Hirose, T. Yoshida, Y. Kobayashi, Y. Sato and K. Koizumi, *J. Chromatogr.*, 485 (1989) 432.