Computer Simulation as a Means of Developing an Optimized Reversed-Phase Gradient-Elution Separation

J. W. Dolan / L. R. Snyder*

LC Resources Inc., 3182C Old Tunnel Rd, Lafayette, CA 94549, USA

M. A. Quarry

Medical Products Dept., E. I. Du Pont de Nemours & Co., Wilmington, DE 19898, USA

Key Words

Gradient elution
Reversed-phase
Method development
Computer simulation
Retention prediction

Summary

A personal-computer program (DryLab G) is described for the simulation of reversed-phase gradient-elution separations. After two experimental gradient runs are carried out initially, this program allows the user to develop a final separation by varying gradient conditions (gradient time, initial and final %-organic in the mobile phase, gradient shape), column dimensions, flowrate and particle size. This approach takes advantage of "solvent-strength selectivity", as reported recently [28] for isocratic separations. Method development using this procedure can result in better separations with much less effort. Examples of its validation and application are presented.

Introduction

Gradient elution is useful for the HPLC analysis of many samples. Mixtures with a wide retention range cannot be handled effectively by isocratic separation, but the use of gradient elution is not restricted to such cases. Sometimes a sample with a limited retention range can be eluted under isocratic conditions, but will nevertheless be better separated by gradient elution. In the past, for various reasons, many workers have been reluctant to accept gradient elution methods. However this situation is now changing, and in the future gradient elution is expected to be used routinely in most laboratories.

The general principles required for developing and optimizing a gradient elution method are now well known [1–4]. However it is doubtful that the full potential of gradient elution is very often realized in practice. The technique is inherently complex, and many different separation variables are involved. As a result, changes in separation conditions not infrequently lead to surprising results and a need for additional experiments. Samples that require gradient elution often contain a larger number of individual components, and this also adds to the difficulty of systematic method development. Finally, the effective use of gradient elution should take advantage of changes in band spacing (variations in α) that can result from a change in gradient conditions (apart from other changes in the HPLC system [4–8]).

A rigorous mathematical description of gradient-elution retention as a function of separation conditions has been available for some time [2–4]. Bandwidth in gradient elution can also be predicted for the various HPLC methods (reversed-phase, ion-exchange, etc.) for different separation conditions, including the nature of the sample and the type of column used [2–4, 9–11]. In principle, it is therefore possible to predict the resolution obtainable in a given separation as a function of such variables as gradient time, initial and final mobile phase composition, flowrate, column dimensions, etc. For a given HPLC system, however, it is necessary to first measure the dependence of isocratic retention on mobile phase composition, for each solute in the sample.

The latter approach presumes the use of a computer in carrying out the necessary calculations for each simulated run (with some change in conditions). Several examples of this type have been reported [12–15]. So far, however, these cases have involved the application of computer-simulation to specific problems. No effort has been made to develop an overall strategy built around computer-simulation as the main tool for developing a gradient-elution method. Also, previous examples of the prediction of separation in gradient elution often lack the accuracy required for method development.

0009-5893/87 0261-16 $ 03.00/0 © 1987 Friedr. Vieweg & Sohn Verlagsgesellschaft mbH

Commercial software (DryLab G) has recently become available for predicting separation as a function of experimental conditions in reversed-phase HPLC carried out in a gradient mode. Here we describe some preliminary examples which illustrate the potential of this approach for developing and optimizing a final gradient-elution method. We will also explore some possible problems that can arise during computer simulation, especially for the case of unknown samples that contain a large number of components.

Theory

Resolution in both isocratic and gradient elution is given as

$$R_s = 2(t_2 - t_1)/(W_1 + W_2). \quad (1)$$

Here t_1 and t_2 refer to the retention times t_g of adjacent bands 1 and 2 (sec), and W_1 and W_2 are their baseline bandwidths W (sec). A prediction of resolution therefore requires values of t_g and W for each band in the sample.

Prediction of Retention Times t_g

Retention time in gradient elution is given as (2)

$$\int_0^{t_g} F\, dt/[V_m(1 + k')] = 1 \quad (2)$$

Here F is the mobile-phase flowrate, V_m is the column dead-volume (equal to F t_0), and k' is the capacity factor of the solute at time t during the gradient. For the case of more complex gradients, those composed of several segments (including pre-elution by the initial mobile phase and post-elution by the final mobile phase), values of t_g can be determined by applying an alternative form of eq. (2) to each gradient segment (pp. 135–42 of Ref. [3]) and combining the results.

The preceding procedure is greatly simplified if an explicit relationship is assumed between capacity factor values k' and mobile phase composition. Numerous studies (see discussion of Ref. [16, 17]) have confirmed that values of k' in reversed-phase HPLC are related approximately to the volume-fraction of organic solvent in the mobile phase (ϕ) as

$$\log k' = \log k_w - S\phi. \quad (3)$$

Here, S is a constant for a given solute, and k_w is the value of k' for $\phi = 0$. This relationship is assumed here as a first approximation, but predicted values of t_g can be corrected for small curvature of plots of $\log k'$ vs ϕ [18].

Estimates of values of k_w and S. Previous attempts to use eq. (1) for the purpose of predicting values of t_g in gradient elution usually assume that values of k' vs ϕ have been determined experimentally from isocratic measurements. However this is experimentally inconvenient, because samples that require gradient elution are not easily studied by isocratic elution. A better approach is to carry out two initial gradient runs and use these to obtain values of $\bar{k}$ and S as described previously [19] for the prediction of isocratic retention as a function of ϕ. This same procedure can be used for predicting t_g values in gradient elution as a function of experimental conditions.

When pre-elution and post-elution of the solute in gradient elution can be ignored, values of $\bar{k}$ and S can be obtained from the numerical solution of two simultaneous equations [19]:

$$(t_g)_i = (t_0/b_i)\log(2.3k_0 b_i + 1) + t_0 + t_D \quad (4a)$$

and

$$(t_g)_j = (t_0/b_j)\log(2.3k_0 b_j + 1) + t_0 + t_D. \quad (4b)$$

Here $(t_g)_i$ and $(t_g)_j$ are experimental retention times for a given solute obtained from runs i and j with linear gradients of different steepness: $b = b_i$ and b_j. The quantity t_D is the dwell-time of the HPLC gradient equipment, equal to the time it takes the gradient to move from the mixer to the column inlet. The gradient steepness parameter b is given as

$$b = V_m \Delta\phi\, S/t_G\, F, \quad (5)$$

where $\Delta\phi$ refers to the change in ϕ during the gradient, t_G is gradient time, and S is given by eq. (3). The quantity k_0 refers to the original value of k' at the start of the gradient; it is given by

$$\log k_0 = \log k_w - S\phi_0, \quad (6)$$

where ϕ_0 is the value of ϕ at the start of the gradient.

The above procedure becomes more complicated when the initial gradient runs are affected by pre-elution of the sample, as can occur when the dwell time t_D is fairly large, and values of k_0 are small (see discussion of Ref. [20]). In principle this problem could be handled by techniques for the numerical solution of eq. (1) as applied to the combined gradient, which includes pre-elution followed by gradient elution. An example of this approach is suggested by the work of Parente and Wetlaufer [21], for the prediction of corresponding isocratic parameters in ion exchange. However this procedure can be complex as well as time consuming; we wanted a method that would yield final values of k_w and S within short run-times on a personal computer such as the IBM PC. We were able to achieve this by an iterative solution based on the fact that S-values obtained from eqs. (4a, b) are relatively insensitive to pre-elution. This procedure is incorporated into the DryLab G software used in the present study.

Prediction of Bandwidth W

Elsewhere [4] it has been shown that bandwidth in gradient elution is given as

$$W = t_0(1 + k_f)\, J\, G\, N^{-1/2}, \quad (7)$$

where k_f is the value of k' at the time the band leaves the column, N is the column plate number, G is a gradient "compression factor" and J is a correction factor that accounts for an anomalous broadening of bands in gradient elution when the gradient is quite steep [9]. If experimental values of J are multiplied by calculated values of G, it is

found that the product

$$JG \approx 1.1 \pm 0.1 \quad (8)$$

over the entire range of values of b. This allows eq. (7) to be restated approximately as

$$W = 1.1 t_0 (1 + k_f) N^{-1/2}. \quad (9)$$

Eq. (9) has been found by others [3] to be in approximate agreement with experimental data from a number of gradient HPLC systems. The value of k' when a band leaves the column during gradient elution is given as [2]

$$k_f = 1/[2.3b + (1/k_0)]. \quad (10)$$

Value of N in gradient elution. The quantity N in eq. (9) is the plate number measured in a "corresponding" isocratic system, where the effective value of k' in gradient elution ($\bar{k}$, equal to k' when the band is at the column midpoint) is equal to k' in isocratic elution for the same column and similar other conditions. Values of N can be predicted as described elsewhere [4, 22, 23], when the chromatographic conditions are specified and the molecular weight of each solute can be estimated.

Control of Resolution in Gradient Elution

Resolution in gradient elution can be expressed as a function of the same factors that are used to control resolution in isocratic separation; i.e., capacity factor k', plate number N and separation factor α:

$$R_s = (1/4)(\alpha - 1) N^{1/2} [\bar{k}/(1 + \bar{k})]. \quad (11)$$

Previous discussions of resolution in gradient elution [1, 2, 4, 24, 25] have emphasized the importance of the column plate-number N and the effective capacity factor $\bar{k}$. Systematic changes in gradient conditions that affect N and $\bar{k}$ can be used to increase the peak capacity of a gradient separation and maximize overall resolution.

We believe that changes in band spacing (values of α) are of equal importance in optimizing separations by gradient elution. All the separation variables used to control values of α in isocratic elution can be used in the same way for gradient elution: a change in the organic solvent, changes in pH or temperature, a change in the column stationary phase, etc. (1, and see specific examples of Ref. [14, 15, 26] for gradient elution).

During the initial phase of method development for gradient elution, we have found that changes in α are conveniently achieved by varying gradient conditions so as to vary $\bar{k}$ [5, 8], which is given as

$$\bar{k} = 1/1.15b. \quad (12)$$

Corresponding changes in band spacing are also observed in isocratic elution as k' or ϕ is varied ([27, 28], and see especially discussion of Ref. [29]). Previously we have shown that it is possible to systematically vary gradient-elution band-spacing in this way by changing either flow-rate or gradient time. Here we will show that similar changes in band spacing can also result from changes in ϕ or the use of complex gradients (linear segments of different steepness). This in turn provides a basis for a considerable control over band spacing in gradient elution.

Experimental

Equipment, Materials and Procedures

The HPLC system was a DuPont 8800 liquid chromatograph equipped with a Model 860 fixed-wavelength (254 nm) detector and column oven. Procedures and solvents are described in Ref. [20].

Samples. The polyaromatic hydrocarbon mixture was supplied by Supelco Inc. (Bellefont, PA). The herbicide mixture was obtained from the Agricultural Chemicals Dept. of DuPont (Wilmington, DE, USA), and is described further in Ref. [29]; several of these compounds are proprietary in nature, and their exact composition was not disclosed to us.

Computer Simulations

All simulations were carried out on an IBM XT personal computer, using DryLab G software (LC Resources Inc., Lafayette, CA, USA).

Results and Discussion

The application of a somewhat complex scheme for the computer simulation of gradient-elution separation first requires validation of the overall procedure. Then a systematic approach based on computer simulation must be designed for the development of optimized gradient elution methods. This approach will differ from that used in experimental trial-and-error procedures (the traditional way in which gradient methods have been developed), for reasons that will become apparent. Next, possible problems that can arise in the computer-simulation approach should be considered, and remedies to these problems must be found. Finally, actual applications of the overall scheme to real samples are required – as a practical demonstration of the value of such an approach. The present paper will address each of these areas.

Accuracy in the Prediction of Retention in Gradient Elution

Previous studies [2–4, 30, 31] have reported on the accuracy of predictions of gradient retention t_g as a function of values of k_w and S obtained from corresponding isocratic runs. Here we are interested in the accuracy of t_g-values predicted from k_w and S values derived from initial gradient runs. Because these data are to be used mainly for predictions of resolution (eq. (1)), the accuracy of relative retention ($t_2 - t_1$) is of greater interest than the accuracy of individual t_g-values. The present software (DryLab G) is also designed to correct for pre-elution effects in (a) the gradient data from which values of k_w and S are initially derived and (b) t_g-values that are to be predicted by computer-simulation. We have therefore tested these specific cases. Suitable data for the present validation of the DryLab G software were either (a) obtained from the literature or (b) measured by us as reported here.

Phthalate ester sample. Gradient retention data have been reported [31] for five C_1–C_5 dialkylphthalates under various conditions. DryLab G requires starting data for two

runs carried out with different t_G values; gradient times of 20 and 80 min were selected for this purpose from the data of Ref. [31] (other conditions the same). In selecting values of t_G for the two starting gradient runs, it is advisable that these t_G-values differ by a factor of 3 or more for maximum accuracy in the resulting predictions of t_g for other gradient conditions [19]. With these two experimental runs as input to DryLab G, computer simulation was then carried out for other gradient times, as summarized in Table I.

The experimental test of computer-simulation summarized in Table I corresponds to near-ideal experimental conditions. All bands (except one solute in one run) elute during the gradient, with negligible contribution of pre-elution effects during initial elution. Furthermore, special care was taken in this study to evaluate the usual contributions of equipment and other factors to non-ideal gradient behavior (see discussion of [20, 31]). As a result, we should see good agreement between the experimental and calculated values of t_g in Table I. Maximum accuracy in such predictions will occur for interpolated conditions ($20 < t_G < 80$ min), and for this case (Table I, $t_G = 40$ min) we see that both retention time and resolution (R_s is proportional to $[t_2 - t_1]$) are predicted within about ± 1% (1 SD). For other values of t_G (5, 10, 160, 320 min), the prediction of retention time is accurate within an average error of ± 6% (1 SD), while resolution is accurate within ± 5%. This is adequate for the purposes of using computer-simulation in lieu of actual gradient runs during method development.

The agreement between experimental and computer-simulated values of t_g and resolution in Table I is superior to that found for corresponding isocratic values predicted from the same experimental gradient data [19]. That is, experimental gradient data seem better suited for predicting t_g values in gradient elution, than for predicting isocratic retention as a function of ϕ. We have seen other instances where both this and the converse are true (i.e., isocratic data are better suited for predicting isocratic retention as a function of ϕ). From a practical standpoint, when the highest accuracy in computer-simulated retention or resolution data are required, it suggests using experimental runs that are matched to the same technique as assumed for the computer simulations (isocratic/isocratic or gradient/gradient).

Steroid sample. Gradient separations were carried out with a 6-component steroid sample, varying both gradient time and range. Separations for a 5–100% methanol/water gradient and t_G-values of 30 and 90 min are shown in Fig. 1, with retention data superimposed on these plots. Additional runs were carried out, varying not only gradient time but the initial and final ϕ-values for the gradient. This provides an additional test of the DryLab G software for retention predictions when substantial pre-elution of the solute occurs (see Table II).

It is seen in Table II that retention times t_g are predicted with an average error of ± 3% (1 SD), while resolution (values of $t_2 - t_1$) are accurate with an average error of ± 7% (1 SD). This is adequate for purposes of method development. The data of Fig. 1 and Table II can also

Table I. Comparison of experimental and computer-simulation values of t_g in the gradient separation of five dialkyl-phthalates. Experimental data of Ref. [31], computer simulations using DryLab G. 25-cm column of Zorbax® ODS (6-nm pore), 10–100% acetonitrile/water gradients, 35°C, dwell volume $V_D = 5.5$ mL, data for $t_G = 20$ and 80 min used as inputs to DryLab G.

$t_G = 5$ min

Solute	t_g (min)		$(t_2 - t_1)$ (min)		% error	
	expt.	calc.	expt.	calc.	t_g	$(t_2 - t_1)$
C_1	6.88	6.78	0.78	0.74	−2	− 5
C_2	7.66	7.52	0.28	0.29	−2	+ 3
C_3	7.94	7.81	1.13	1.02	−2	−10
C_4	9.07	8.83	0.68	0.45*	−3	−
C_5	9.75	9.28*			−	
			avg error (1 SD)		−2 ± 0	−4 ± 8

Solute	t_g for indicated value of t_G							
	$t_G = 10$ min		$t_G = 40$ min		$t_G = 160$ min		$t_G = 320$ min	
	expt.	calc.	expt.	calc.	expt.	calc.	expt.	calc.
C_1	8.82	8.71	16.20	16.41	32.0	30.9	43.8	39.7
C_2	10.31	10.17	21.72	21.94	51.7	50.1	79.8	72.7
C_3	10.92	10.79	24.45	24.65	62.9	61.3	102.1	94.5
C_4	12.83	12.75	31.76	31.92	89.7	87.8	153.5	144.3
C_5	13.74	13.68	35.80	35.86	105.4	104.2	183.6	177.5
error (1 SD)								
t_g	−1 ± 0%		+1 ± 0%		−3 ± 3%		−7 ± 3%	
$(t_2 - t_1)$	0 ± 2		−1 ± 1		0 ± 3		+0 ± 8	

* DryLab G indicated that this band elutes after the gradient is over; while DryLab G corrects for such post-elution, the rounding of the gradient near its end is quite severe for small values of t_G (see discussion of Ref. [18, 30]), leading to greater error in the final value of t_g predicted by computer-simulation

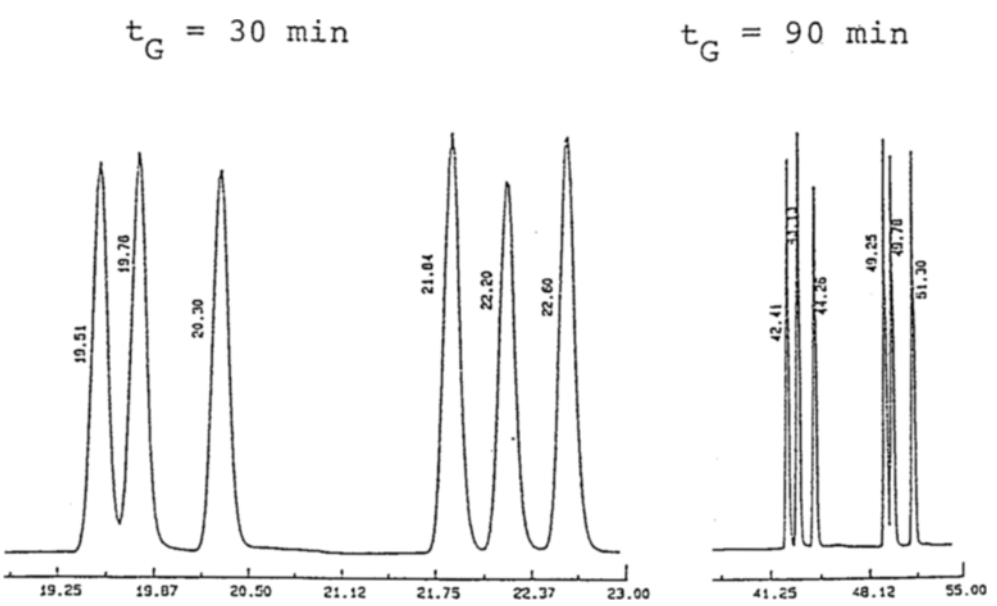


Fig. 1

Gradient separations of a steroid sample with two different values of t_G. Data used for calculations of Table II. Column, 25 X 0.46-cm Zorbax® ODS; 5–100% methanol/water gradients; flowrate, 2mL/min; 35°C, dwell volume V_D = 5.5mL. Sample components, in order of elution (#1–6 of Table II): prednisone (band 1), cortisone (2), hydrocortisone (3), dexamethasone (4), corticosterone (5), cortexolone (6)

Table II. Comparison of experimental and computer-simulation values of t_g in the gradient separation of six steroids. Experimental runs of Fig. 1 used as input to DryLab G. Other conditions the same, except for gradient time t_g and gradient range.

Solute*	t_g for indicated values of t_g and gradient range (min)							
	t_g = 15min 5–100% MeOH**		t_g = 45min 5–100% MeOH**		t_g = 30min 35–75% MeOH**		t_g = 15min 55–75% MeOH**	
	expt.	calc.	expt.	calc.	expt.	calc.	expt.	calc.
1	12.71	12.62	25.83	25.75	13.51	13.85	3.86	3.46#
2	12.84	12.75	26.20	26.12	14.04	14.38	4.08	3.70#
3	13.18	13.07	26.82	26.84	15.05	15.38	4.71	4.43##
4	13.89	13.80	29.29	29.22	18.72	18.96	6.44	6.37##
5	14.13	14.04	29.72	29.66	19.23	19.52	7.00	6.99##
6	14.29	14.20	30.38	30.32	20.33	20.58	7.56	7.60##
error (1SD)								
t_g	+1 ± 0%		0 ± 0%		−2 ± 1%		+5 ± 5%	
$(t_2 - t_1)$	+1 ± 5		+1 ± 5		+3 ± 6		+8 ± 9	

* numbered in order of elution; see Figure 1 for identity (no band reversals)

** MeOH refers to methanol

\# these bands elute isocratically in 55% MeOH/water (before start of gradient)

\#\# these bands exhibit pre-elution; fractional migration in initial mobile phase (55% MeOH) is: band #3, 79%; band #4, 46%; band #5, 40%; band #6, 35%; i.e., band #3 migrates 79% through the column before the initial gradient reaches it.

be used to illustrate another practical point. Generally it is advantageous to use larger values of t_g (or $\bar{k}$) in the experimental runs used for computer simulation. Thus, if the 15 and 45-min runs of Table II are used as input for computer simulation, the resulting errors in predicted resolution (for the remaining runs of Fig. 1 and Table II) are significantly larger: ± 11% (1SD), or 50% greater error than for the predicted values of Table II[1]. This is discussed in a later section.

[1] In this case (15/45-min runs) the predicted values of t_g are in error by only ± 2% (1SD), which is misleading if considered in isolation. That is, accuracy in values of $(t_2 - t_1)$ is of major concern, since this determines the accuracy of predictions of resolution for various experimental conditions.

Additional examples of the accuracy of computer simulation for predicting values of t_g will be found in the following discussion.

A Systematic Approach to Method Development for Gradient-elution HPLC, Based on Computer-simulations

With the advent of computer simulation as an alternative to trial-and-error experimental runs, it is possible to develop and improve a gradient separation in the same way as previously – where actual experimental runs were used to evaluate separation vs a change in conditions. Computer simulation requires a small fraction of the time required for actual runs, so many more possibilities can be explored. However a computer offers additional features that we will illustrate here. This in turn suggests the approach to systematic method development outlined in Table III, which has evolved as a result of our experience with the DryLab G program. We will illustrate this procedure with several examples.

Mixture of OPA-Amino Acids

Eslami et al. [32] have reported data for the separation of 22 o-phthaldehyde (OPA) amino acids by reversed-phase HPLC (17–55% acetonitrile/water linear gradients). These data (32 runs involving different t_G values and 5 different column lengths) were made available to us for evaluation using DryLab G. In the following example, we have assumed that an optimum method is to be developed, and have used those data that were appropriate to this goal. We will follow the 6 steps of Table III.

Step 1. Two gradients were run initially, with the results shown in Figs. 2a, b. The gradient times are (a) t_G = 15 min and (b) t_G = 35 min. It is seen that there are 22 distinct bands in each chromatogram; i.e., no unresolved band pairs. DryLab G requires that each band in the second run (b) be numbered to correspond to band-sequence in the first run; i.e., the band labeled #1 must be the same compound in each run, even if there are band reversals between the two runs. We will discuss this further in a following section. It is seen that there are no band reversals in run (b), so retention times for each run are entered in sequence.

Step 2. These retention data and other separation conditions (see Fig. 2) were entered into the computer, and a relative-resolution map (RRM) was requested, as shown in Fig. 3. A RRM is a plot of the resolution of the poorest-resolved band-pair in the sample vs gradient time t_G, assuming a 10,000-plate column (N = 10,000) [27, 28]. It allows a quick conclusion as to the optimum gradient time for a given separation. Note that the poorest-resolved band-pair is indicated on the RRM; this is seen to be bands #9/10 for $t_G < 5$ min, and bands #6/7 for $t_G > 5$ min. The maximum resolution possible for this sample, using the same column and flowrate, is about $R_s = 1$ for a 10,000-plate column. That is, this is a fairly difficult separation. We also see that there are no band-reversals as t_G is varied; i.e., there is little change in separation selectivity as a function of t_G or $\bar{k}$. This is somewhat unusual, as will be seen from later examples.

Step 3. The resolution of bands #6/7 in Fig. 2a is about 0.94, estimated from the valley between the two bands (see Table IV). A plate number for the column under these conditions (15 min gradient) was next requested. DryLab G estimates this value of N (N = 11,600 in this example) from the known retention times and resolu-

Table III. Recommended approach to HPLC method development using gradient elution, based on computer simulation with DryLab G.

Step	Goal
1. carry out two experimental runs with 5–100% organic/water gradients, and t_g values of 30 and 90 min*	input retention data into computer to allow predictions of separation as a function of varying conditions
2. request a relative-resolution map (RRM) as a function of t_g	determine feasibility of sample separation with present HPLC system**; determine optimum t_G-value (preliminary)
3. adjust resolution to match one of initial experimental runs (optional)***	obtain exact resolution values for computer simulations with original column and flowrate
4. determine optimum gradient range	minimize run time
5. fine-tune band-spacing and resolution, by (a) varying initial %-organic in gradient, (b) using segmented (non-linear) gradient	optimize final gradient for resolution and run time
6. vary column conditions while holding gradient conditions fixed	retain optimized band spacing from steps #2–5, while optimizing plate number N; finalize method for adequate resolution, acceptable pressure and minimum run time

* the choice of t_G-values is arbitrary; the two values should differ by a factor of 3 to 4 for accurate predictions, columns with large N-values are preferred (e.g., 25-cm, 5-μm), and flowrates should be 1–2 mL/min

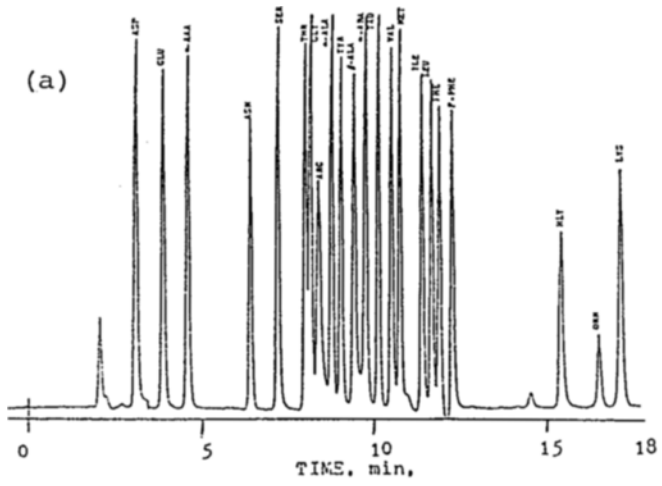
** for the successful development of a final gradient method, the RRM should show $R_s > 1$ for some value of t_G

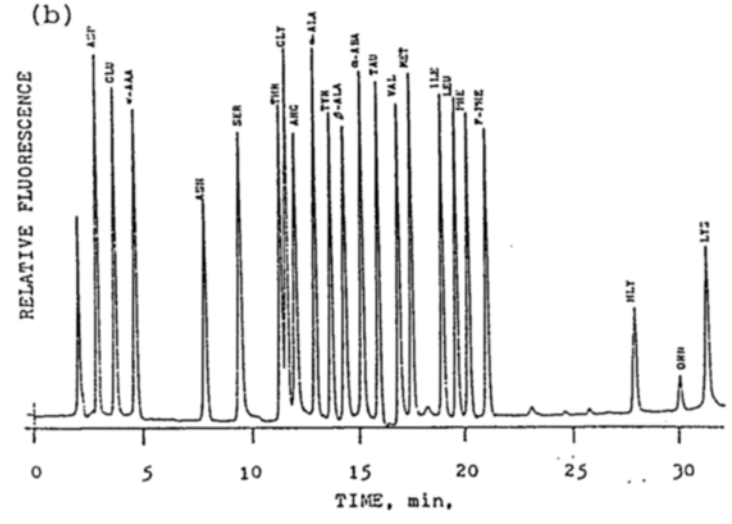
*** this option is mainly useful when column length, particle size and flowrate are to be held constant; for this situation, simulations as a function of gradient conditions will more closely match experimental runs

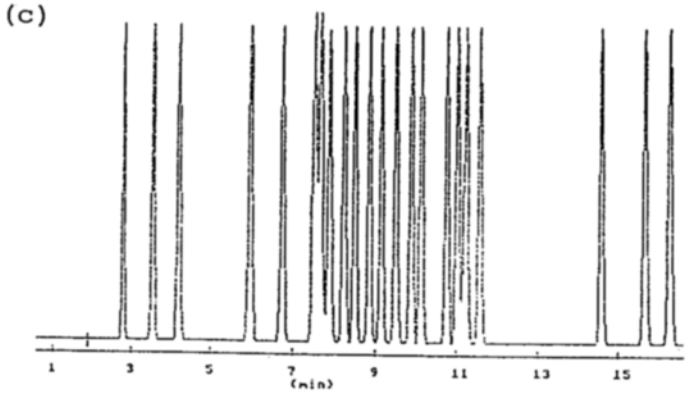
Table IV. Estimating resolution from the valley between two overlapping bands. Calculated for Gaussian bands.

Valley*	R_s for indicated ratio of band sizes				
	1/1	2/1	4/1	8/1	16/1
3%	1.46	1.50			
5	1.35	1.42	1.48	1.52	
8	1.26	1.33	1.40	1.45	1.50
10	1.22	1.29	1.35	1.41	1.47
15	1.14	1.21	1.27	1.33	1.39
20	1.07	1.15	1.21	1.27	1.33
30	0.97	1.06	1.12	1.19	1.24
40	0.90	0.98	1.06	1.12	1.18
50	0.83	0.92	1.00	1.07	1.12
60	0.78	0.87	0.95	1.02	1.08
80	0.68	0.78	0.86	0.93	0.99

* Measured as height of valley from baseline divided by height of smaller band; see Ref. [1], pp. 46–7 for details







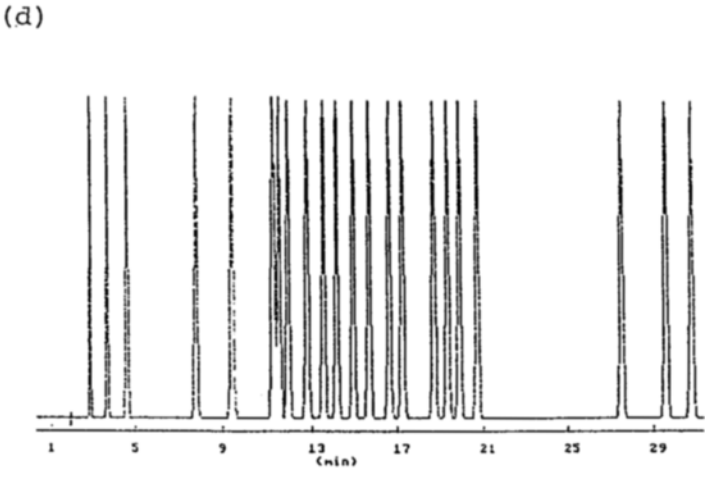


Fig. 2

Gradient separations of a mixture of 22 OPA-derivatives of the common amino acids. Data used for calculations discussed in text and shown in Figs. 6 and 7. Column, 15 × 0.46-cm IBM C18; 17—55 % acetonitrile/water gradients; flow-rate, 0,8 mL/min; 22 °C; system dwell-volume equal to 0.0 mL. Sample components identified in chromatograms. (a) experimental chromatogram, t_G = 15 min; (b) experimental chromatogram, t_G = 35 min; (c) simulated chromatogram, t_G = 15 min; (d) simulated chromatogram, t_G = 35 min.

tion. Selecting this DryLab G option automatically adjusts all resolution calculations for the actual plate number of the column, rather than for an N-value of 10,000 as in Fig. 3. At this point we have the option of simulating the starting runs (Figs. 2a, b), and these simulations are shown in Figs. 2c, d for comparison with the experimental runs (Figs. 2a, b). Good agreement between computer-simulated and actual runs is observed. Returning to Fig. 3, we see that resolution reaches a maximum for t_G = 25–30 min. Under these conditions, the shortest gradient time is desirable, and a value of t_G = 27.5 min was selected.

Step 4. The gradient range (initial and final %-organic in the mobile phase) was next adjusted. The computer will recommend an approximately optimum range if requested: 15–59% acetonitrile/water was predicted in this case. This range is slightly different from that used initially (17–55%), but not significantly so. In this case we opted to keep the original range (17–55%) because experimental data were not available for other ranges.

Step 5. Changes in ϕ_0 or gradient shape were not considered useful in the present example, because of the minimal change in selectivity (band spacing) as t_G and $\bar{k}$ are varied. Trial-and-error changes in these parameters, using computer simulation, confirmed that this was the case.

Step 6. Computer simulations were continued by varying column conditions. DryLab G allows variation of column dimensions, particle size and flowrate. Since only columns of varying length were available in the present data set, a longer column (25 cm) was tried. When column size or flowrate is changed in gradient elution (eq. (5)), the effective gradient steepness b changes also. Since Steps 1–5 are intended to optimize b and sample retention, it is undesirable to vary b when optimizing column conditions. DryLab G therefore automatically adjusts the gradient time t_G to compensate for any changes in column size or flowrate (so as to keep b and relative retention constant). In this case, the use of a longer column (25 cm vs 15 cm to start) requires an increase in t_G by the ratio (25/15), or a final t_G-value of 46 min.

The simulated chromatogram predicted for these gradient conditions is shown in Fig. 4a. Band-pair #6/7 is least resolved, with a predicted R_s = 1.36. The actual chromatogram for these conditions is shown in Fig. 4b; the experimental resolution for bands #6/7 is R_s = 1.5 (Table III). This is reasonable agreement, considering that different columns (15 cm) were used to generate the computer simulation for the 25-cm column.

Retention data for computer-simulated vs experimental runs. Comparison of the simulated and actual chromatograms of Fig. 4 shows an error in predicted retention times of $-2 \pm 1\%$ (1 SD), and an error in $(t_2 - t_1)$ of $3 \pm 9\%$ (1 SD). This compares favorably with the data of Tables I and II, especially when the use of different columns for experimental input and final prediction is considered. When experimental data for the 25-cm column were used for computer simulation (14 and 46-min gradients), similar errors in predicted t_g-values was found for experimental runs with other gradient times:

t_G	error in t_g (1 SD)
8 min	$2 \pm 4\%$
10	$2 \pm 2\%$
18	$-1 \pm 1\%$
26	$-1 \pm 1\%$
34	$-1 \pm 1\%$

This example therefore confirms the accuracy of these DryLab G simulations.

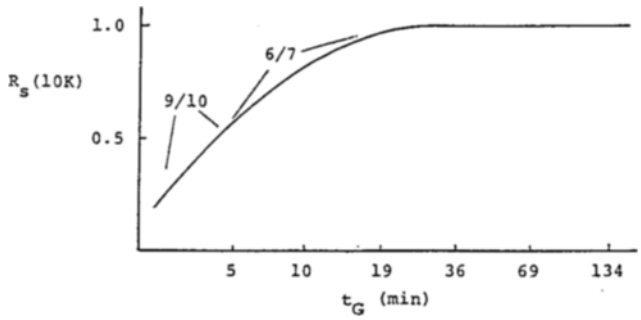


Fig. 3

Relative-resolution Map for OPA-amino acid separations of Fig. 2. Calculated by DryLab G.

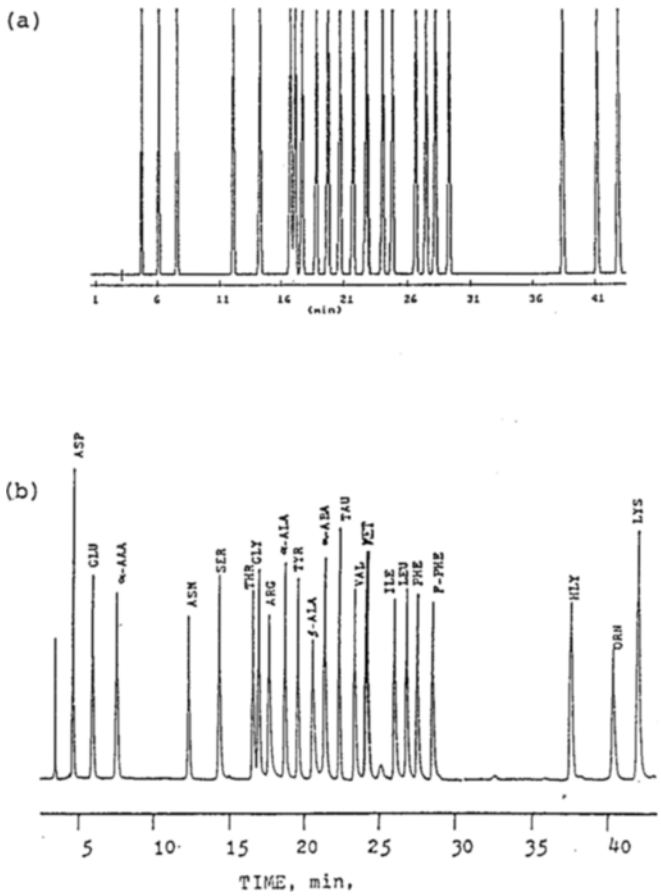


Fig. 4

Optimized separation of OPA-amino acids. Column, 25-cm; gradient time, 46 min; other conditions as in Fig. 2. (a) DryLab G simulation; (b) experimental run.

Mixture of Polyaromatic Hydrocarbons

A sample containing 16 priority pollutants (polyaromatic hydrocarbons, PAHs) required analysis, using reversed-phase gradient elution. Previously a method had been developed for this mixture, using the conventional trial-and-error approach based on experimental runs. The best separation that could be achieved required 17 min for elution of the last band, with a resolution of $R_s = 0.94$ for the poorest-resolved band-pair. Typically the development of such a method would require a minimum of half a dozen runs, and 3 to 5 days of work in the laboratory (depending on the experience of the chromatographer). The design of the DryLab G software makes rapid method development less dependent on operator skill, because each trial-and-error run can be carried out in 2–5% of the time required for an actual run. Computer simulation was next used to repeat the development of this method, using the same column and flowrate employed in the original procedure.

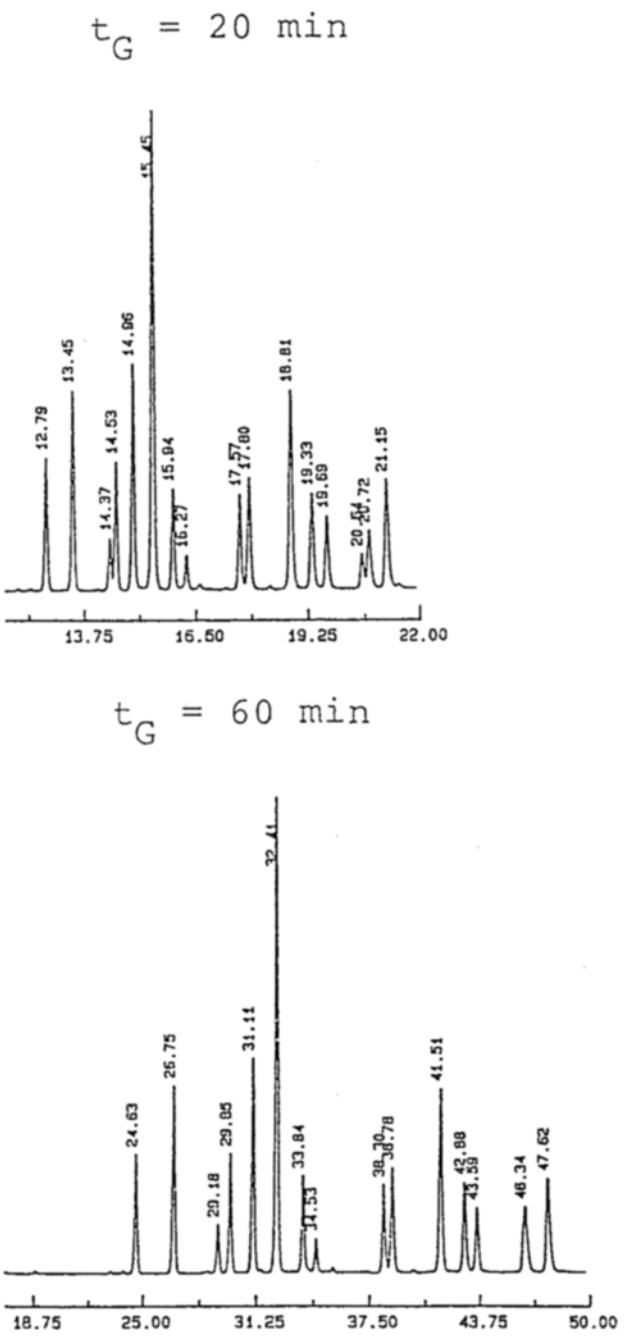


Fig. 5

Gradient separations of a polyaromatic-hydrocarbon sample with two different values of t_G. Data used for calculations discussed in text and shown in Figs. 6 and 7. Column, 15 X 0.46-cm Supelcosil® LC-PAH; 5–100% acetonitrile/water gradients; flowrate, 2 mL/min; 35°C; system dwell-volume equal 5.5 mL. Sample components in order of elution (#1–16) are: naphthalene (1), acenaphthylene (2), acenaphthene (3), fluorene (4), phenanthrene (5), anthracene (6), fluoranthene (7), pyrene (8), benz(a)anthracene (9), chrysene (10), benzo(b)fluoranthene (11), benzo(k)fluoranthene (12), benzo(a)-pyrene (13), dibenz(a,h)anthracene (14), benzo(g,h,i)perylene (15), indeno(1,2,30cd)pyrene (16).

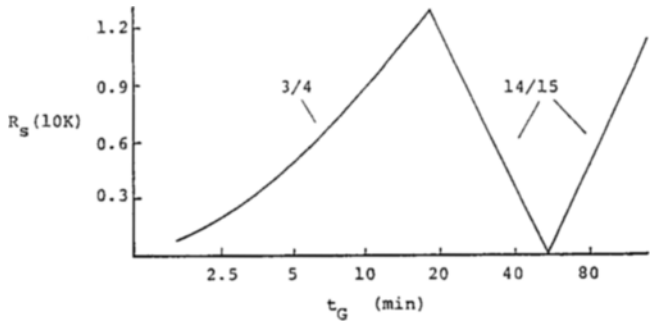


Fig. 6

Relative-resolution Map for PAH separations of Fig. 5. Calculated by DryLab G.

Steps 1 and 2 (Table III). Two gradients were carried out initially, with the results shown in Fig. 5 (step 1). Sixteen bands can be seen in the 20-min gradient run, and 15 bands are present in the 60-min run; bands #14/15 appear to have fused together in the 60-min run. These retention data were entered into the computer, and a relative-resolution map was requested, shown in Fig. 6 (step 2). It appears that a resolution of about $R_s = 1.3$ can be achieved with a 10,000-plate column, so it should be possible to achieve adequate separation of this sample within a reasonable run-time. Band-pair #3/4 limits resolution for $t_G < 20$ min, while band-pair #14/15 limits resolution for $t_G > 20$ min. Bands #3/4 move apart (increase in α) as t_G increases, while bands #14/15 move together with increase in t_G.

Step 3. The resolution of bands #14/15 in the 20-min run of Fig. 5 is about 1.03, estimated from Table IV. A plate number for the column under these conditions was next requested, along with an automatic adjustment of all resolution calculations for a column having this value of N ($N = 7142$). The optimum gradient time from Fig. 6 is about 20 min; a more precise value for this number was next obtained by asking for a summary of R_s vs t_G for $15 \leqslant t \leqslant 27$ min. The best value of t_G determined in this way was $t_G = 19$ min, with a minimum resolution (bands #14/15) of 1.05. This run compares favorably with the original method (slightly better resolution, $R_s = 1.05$ vs 0.94; the run times are essentially the same, 17 vs 19 min.

Step 4. The gradient range was next optimized. Entering a t_G-value of 19 min into the computer, a gradient range of

44–94% acetonitrile/water was recommended by DryLab G. These values were accepted, and an optimum separation was automatically determined by the computer: 44–94% acetonitrile/water with t_G = 10min. The minimum resolution predicted by the computer was R_s = 1.11 (bands #14/15).

Step 5. Finally, a further attempt was made to improve run-time and resolution by fine-tuning the gradient. This can be done by trial-and-error; e.g., simply try different values of ϕ, or investigate segmented-gradients. Often it is useful at the end of step 4 to examine the whole chromatogram as a function of a change in t_G. When this was done for the present sample, it was found that bands #3/4 and 14/15 limit resolution as t_G is varied (as in Fig. 6):

t_G	R_s (3/4)	R_s (14/15)
5min	0.89	1.39
10	1.29	1.11
20	1.47	0.72

These data suggest the use of a 2-step gradient; i.e., a shallow gradient-step (larger $t_G/\Delta\phi$) for the initial separation of bands #3/4 and a steep gradient-step (smaller $t_G/\Delta\phi$) for the final separation of bands #14/15.

Various 2-step gradients were next examined by computer simulation. The first step was selected from 44% acetonitrile starting to a value of 55–70%, ending in a time of 4–8min. The second step was from 55–70% acetonitrile to a value of 100%[2] in a time of 1–4min. The best choice of gradient, on the basis of these simulations was a gradient from 44–60% acetonitrile in 7min, followed by a gradient of 60–100% in 2min (total t_G = 9min).

The resulting computer simulation for this optimum gradient (44/60/100%, 0/7/9min) showed a minimum resolution of 1.26 and a run time of 13min. The computer-simulated chromatogram is shown in Fig. 7b. The conditions for the original method (25/35/100%, 0/2/16min) were also entered into the computer, giving the chromatogram of Fig. 7a. It is seen that the optimum separation of Fig. 7b is faster (13 vs 17min) and resolution is better (R_s = 1.26 vs 1.00 calculated[3]).

More significantly, perhaps, only a half-day of work has been required to this point, including about 30min of computer simulations. Finally, the PAH mixture was run using the final, optimum conditions (44/60/100%, 0/7/9min), with the result shown in Fig. 7c. There is reasonably close agreement between the simulated run of Fig. 7b and the actual run of Fig. 7c, as summarized in the following section.

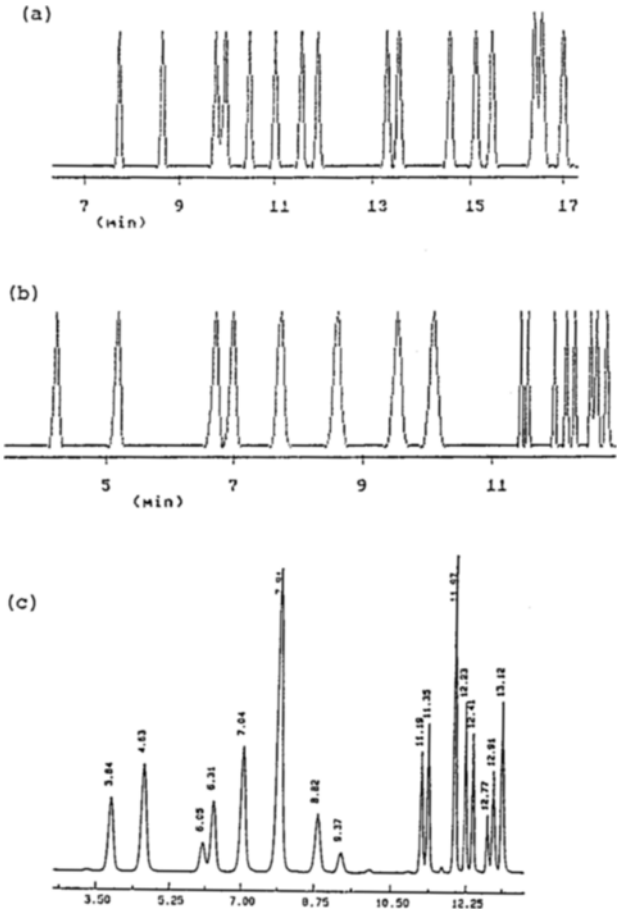


Fig. 7

Chromatograms for gradient-elution separations of PAH sample. Conditions as in Fig. 5, unless stated otherwise. (a) simulation of original PAH method: 2-step gradient, 35/35/100% in times of 0/2/16min; (b) simulation of optimum separation predicted by computer: 2-step gradient, 44/60/100% in times of 0/7/9min; (c) actual separation for same conditions as in (b).

Step 6. Computer simulations were continued by varying column conditions. In this case, only one column was available, so only flowrate variation was studied. Significant improvement in R_s, at the expense of longer run-times, was predicted for lower flowrates. However when actual separations were carried out for flowrates of 1 and 0.5mL/min (t_G values of 18 and 36min, respectively), it was found that resolution became poorer and the bands showed significant tailing. This abnormal behavior was not predicted by DryLab G, and may be an artifact associated with the use of the polymeric bonded phase used in these columns.

Data for computer-simulated vs experimental runs of Fig. 7. A detailed comparison was made between the runs of Figs. 7b, c in terms of retention, bandwidth, and resolution. Our purpose was mainly to verify the accuracy of computer-simulation for complex gradients – in this case a 2-step gradient with significant pre-elution of early bands. The overall agreement between these two runs was satisfactory, but not as good as observed earlier for

2) It might seem logical to end the gradient at the value of 94% acetonitrile initially recommended by the computer. Because the second step is steep, however, it was considered advisable to increase the gradient range as much as possible, in order to minimize effects from the rounding of the gradient by the equipment [20].

3) The calculated value of R_s = 1.00 for the original method can be compared with a value of R_s = 0.94 from the actual chromatogram (not shown).

simple linear gradients. One reason for this is that rounding of the gradient by the equipment [20] is more serious for the case of segmented gradients (vs linear gradients), and is not taken into account by the present version of DryLab G. Errors in the predicted values of various quantities were as follows (averages for 16 bands): retention time t_g, $-4 \pm 3\%$ (1SD); $(t_2 - t_1)$, $10 \pm 11\%$ (1SD); bandwidth, $10 \pm 17\%$ (1SD); resolution, $0 \pm 21\%$ (1SD). Note that the combination of an 11% error in $(t_2 - t_1)$ with a 17% error in bandwidth should yield a 20% error in resolution; this agrees with the observed value ($\pm$ 21%).

Mixture of Herbicides

A sample containing 15 herbicide compounds was to be analyzed by reversed-phase HPLC. Unlike the preceding two samples, which are comprised of mixtures of structurally similar compounds, these herbicides represent a number of different compound types. It was therefore anticipated that band-spacing changes as a function of gradient time t_G would be more pronounced.

Steps 1 and 2 (Table I). Two gradient runs were carried out initially, as shown in Fig. 8. Fifteen distinct bands can be observed in each run, but it is seen that the sequence of bands in the two runs is not identical; bands #8 and 9 reverse their positions in the chromatogram as t_G is changed from 30 to 90min. These retention data (after assignment of the correct band position for each compound) were entered into the computer, and a relative-resolution map was requested (Fig. 9). The RRM of Fig. 9 indicates a much more complex dependence of relative retention on gradient time (and $\bar{k}$) than was found in the previous examples of Figs. 3 and 6. There are four band-reversals as t_G is increased from 5min to 100min. This is illustrated further in the chromatograms of Fig. 10; bands #6 and 8 overlap in the 15-min run, bands #8 and 9 overlap in the 60-min gradient, and bands #2 and 3 are unresolved in the 120-min gradient. Other changes in band position can also be observed in the runs of Figs. 8 and 10.

Step 3. The marginal resolution observed in the RRM of Fig. 9 suggests that we will have to optimize column conditions for a satisfactory final separation. Therefore the empirical adjustment of resolution at this step was omitted. The resolution map of Fig. 9 suggests that an optimum t_G-value (for this gradient range) is about 20min.

Step 4. The gradient range was next optimized. Entering a t_G-value of 20min into the computer, a gradient range of 17–77% acetonitrile/water in a gradient time of 16min was recommended. Resolution (R_s = 1.1min) and run time (16min vs 21min for 5–100% run) were not much improved, however. This simulated run is shown in Fig. 11a.

Step 5. Various attempts were next made to improve the separation by varying the starting %-organic (ϕ_0) and/or using multi-step gradients. The DryLab G software allows a rapid assessment of the value of changes in ϕ_0, accompanied by optimization of t_G. Following a change in ϕ_0, a RRM can be requested so as to optimize t_G. This is illustrated in Fig. 12, for ϕ_0-values of 5, 10, 15 and 20%. Significant changes in resolution are observed as ϕ_0 is varied; the major effect is an increase in R_s for larger t_G-values ($100 < t_G < 250$min) as ϕ_0 is increased. A R_s-value of 1.7 can be obtained with a 20–100% acetonitrile gradient, for a gradient time of 250min. However we found that a better approach was to use a t_G-value corresponding to about 20min in Fig. 9, then optimize column conditions (column length and flowrate). The actual (starting) gradient

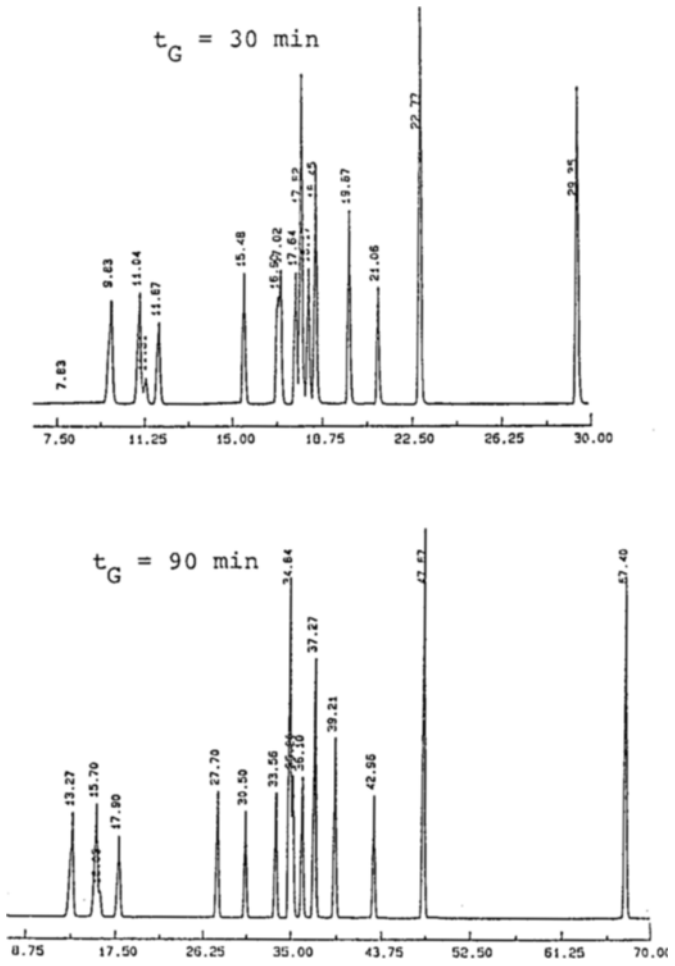


Fig. 8

Gradient separation of a mixture of 15 herbicides with two different values of t_G. Data used for calculations discussed in text and shown in Figs. 9, 11 and 12. Column, 25 X 0.46-cm Zorbax® C8; 5–100% acetonitrile/water gradients; flowrate, 2mL/min; 35°C; system dwell-volume 5.5mL. Sample components described in Ref. [28].

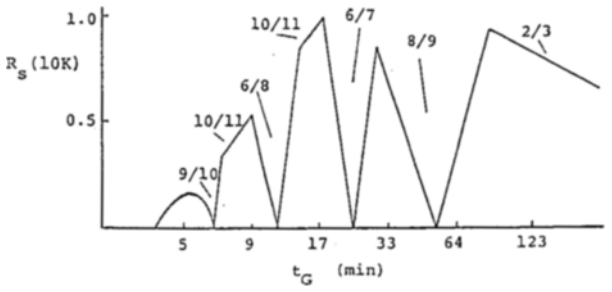


Fig. 9

Relative-resolution map for herbicide separation of Fig. 8. Calculated by DryLab G.

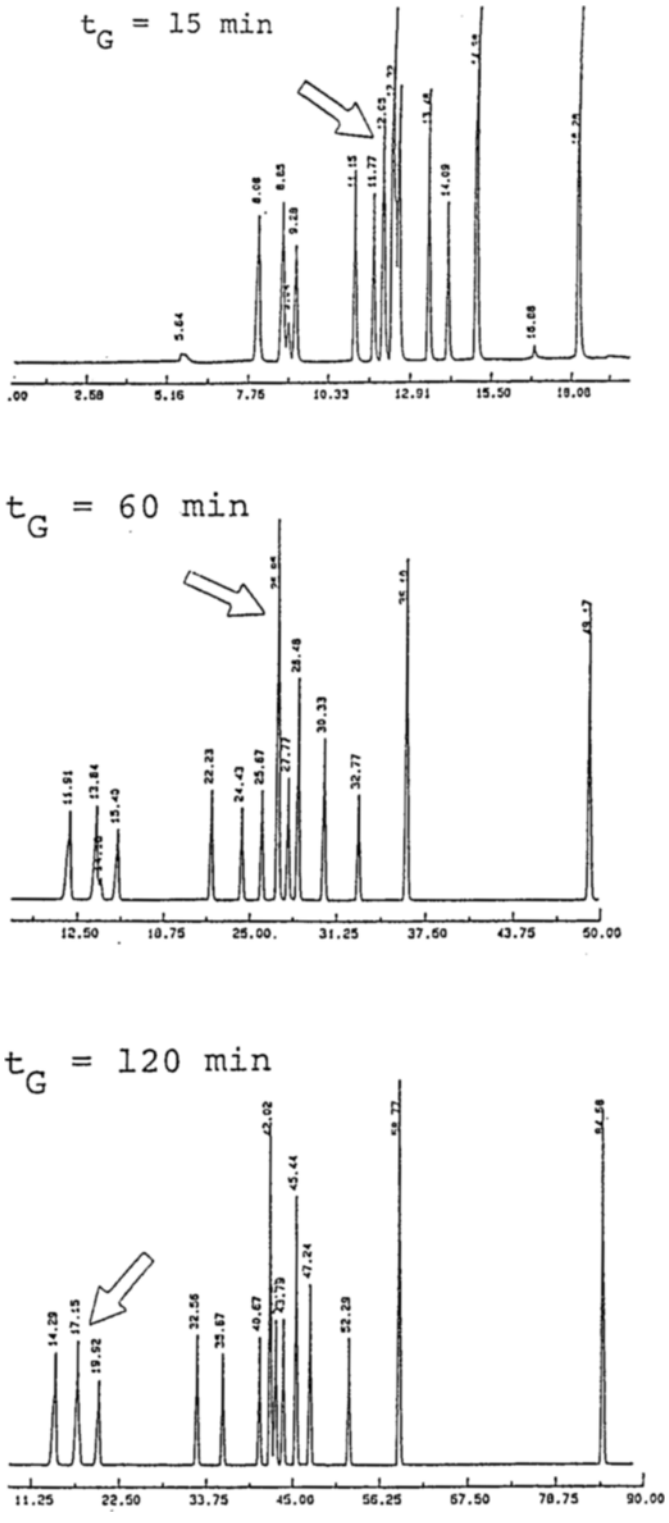


Fig. 10

Gradient separations of herbicide mixture. Conditions as in Fig. 8, except for gradient time (15, 60 and 120min — as indicated). Arrows indicate unresolved band-pairs.

used in Step #6 was a 10–100% acetonitrile gradient with t_G equal 20min.

Multi-step gradients were also studied by computer simulation, but these did not appear to improve resolution significantly. The problem is that bands #6–11 (Fig. 8a) are crowded together in the center of the chromatogram, and exhibit marked changes in band spacing as gradient shape or ϕ_0 are varied. Under these circumstances it is usually preferable to increase the peak capacity of the separation, by increasing the column plate number N.

Step 6. Computer simulations were continued by varying column length and flowrate, using the 10–100% gradient and a gradient time of 20min as starting point (25-cm column, 2mL/min flowrate). An increase in column length by 2-fold (to 50cm) combined with a decrease in flowrate to 1mL/min (from 2mL/min) gave the predicted chromatogram of Fig. 12b (N = 29,900). This was deemed acceptable, and an experimental run was carried out (Fig. 12c). The final chromatogram of Fig. 12c shows good

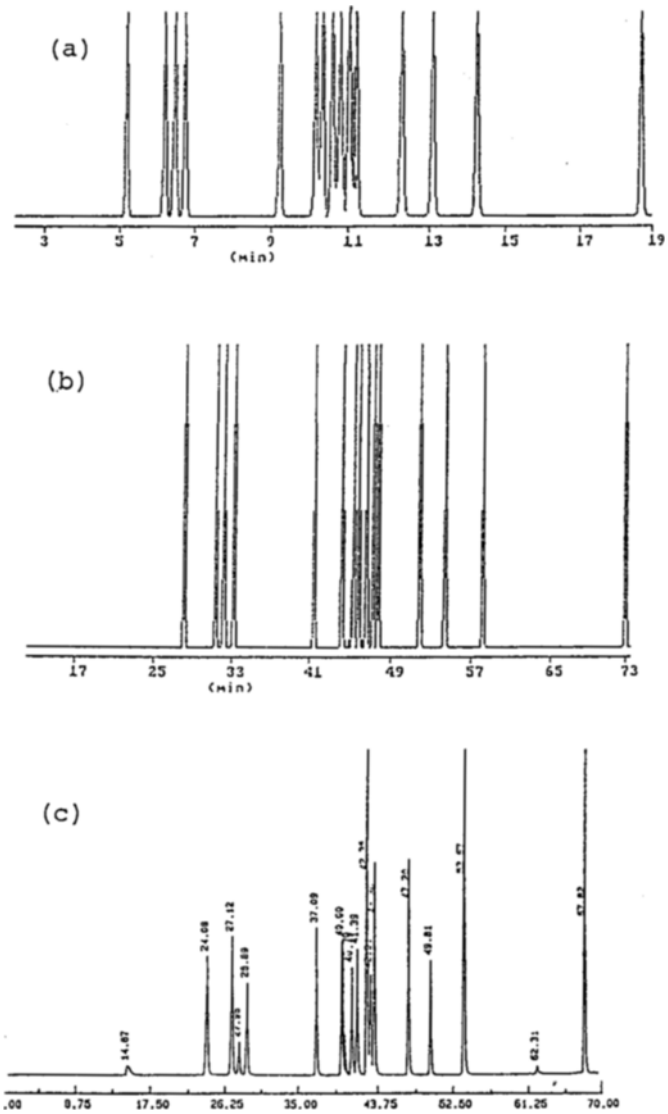


Fig. 11

Optimized separations of herbicide sample. Conditions as in Fig. 8 except where noted otherwise. (a) simulated run for t_G = 16min, gradient range equal 17–77% acetonitrile; (b) simulated run for t_G = 80min; gradient range = 10–100% acetonitrile, 50-cm column and 1mL/min; (c) experimental run corresponding to (b).

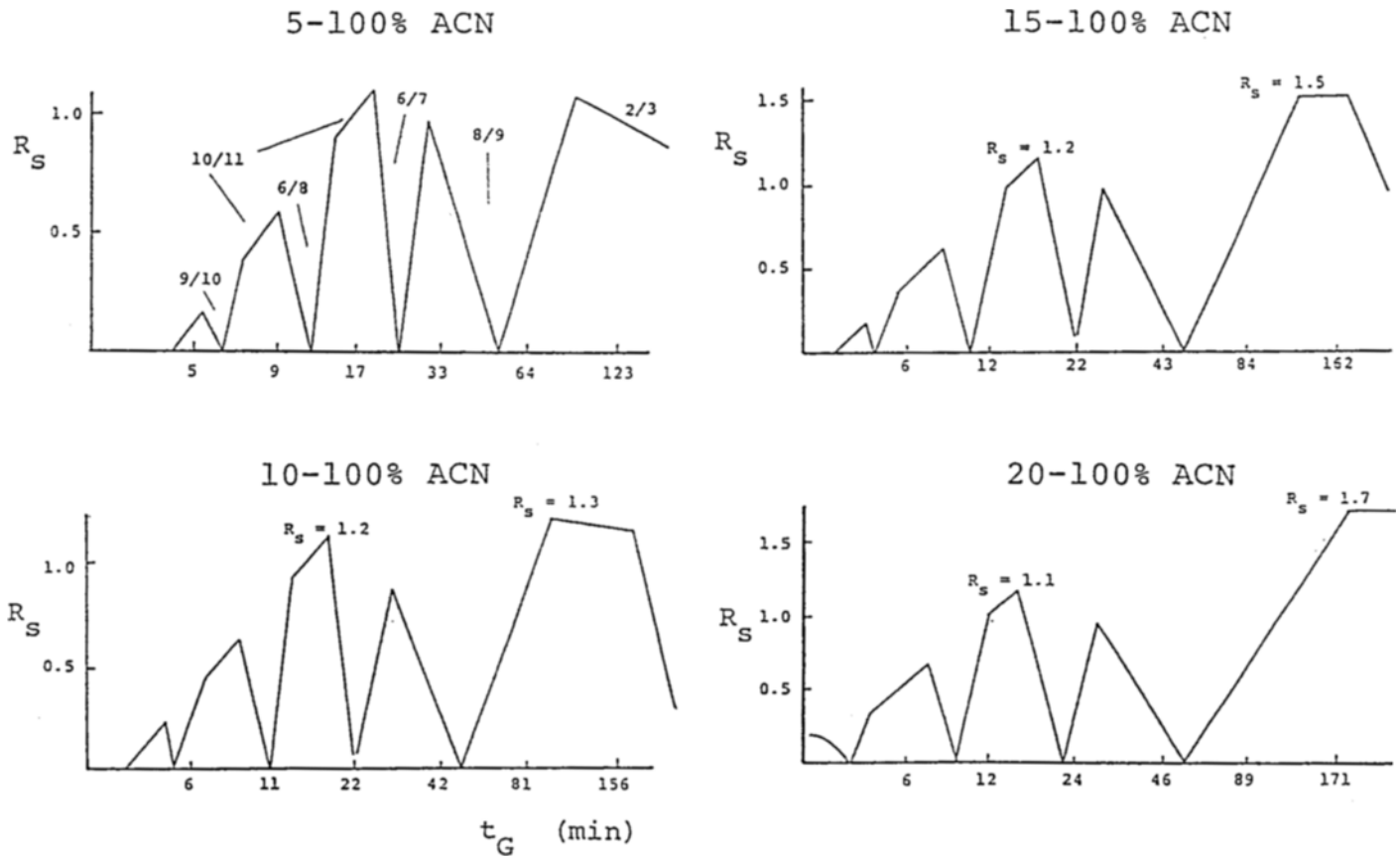


Fig. 12

Resolution maps (N = 11,900) for herbicide sample, using different gradient ranges (indicated in figure).

resolution of all band-pairs and agrees well with the computer simulation of Fig. 12b.

Data for computer-simulated vs experimental runs of Figs. 10 and 12. For the chromatograms of Fig. 10, errors (1SD) in values of t_g and $(t_2 - t_1)$ were as follows: 15-min run, $-2 \pm 0\%$ (t_g) and $-4 \pm 4\%$ $(t_2 - t_1)$; 60-min run, $+1 \pm 1\%$ (t_g), and $+2 \pm 11\%$ $(t_2 - t_1)$; 120-min run, $-1 \pm 1\%$ (t_g), and $-1 \pm 2\%$ $(t_2 - t_1)$.

The corresponding comparison for the experimental run of Fig. 12c is: $+3 \pm 1\%$ (1SD) error in t_g; $+2 \pm 11$ (1SD) error in $(t_2 - t_1)$. The average baseline-bandwidth value for this run was 0.36min vs 0.32min predicted. A similar comparison was made for a 10—100% acetonitrile gradient in a time of 20min, with a 25-cm column and a flowrate of 2mL/min (original column conditions of Fig. 8); the average bandwidth was 0.15min (expt) vs a simulated value of 0.14min.

These various results for the herbicide sample compare favorably with the accuracy of predicted values of retention, bandwidth and resolution presented earlier.

Possible Pitfalls in the Use of Computer Simulation for Predicting Gradient Separation

Gradient elution is often used to separate complex, unknown mixtures which contain numerous components (e.g., 20 or more compounds) of widely varying concentration. This is particularly true for samples of biological origin, such as protein digests, fermentation broths, etc. The use of computer simulation with such samples requires special care if useful results are to be obtained. Other samples of less complexity can also give problems when computer simulation is used for method development. Table V summarizes some specific situations that can contribute to unreliable predictions using DryLab G or similar software.

Mismatched bands. We have noted that each band in the second experimental run used as input for computer simulation must be matched to a corresponding band in the first run. For example, in Fig. 8, band #8 in the 30-min run corresponds to band #9 in the 90-min run. This particular assignment is relatively easy, based on a visual matching of bands according to retention and peak size. In other cases, a similar attempt at matching bands between two runs may result in ambiguous assignments — particularly when several bands of similar size are found in one part of the chromatogram. In these cases, the best approach is usually to run a third experimental run, with a gradient time intermediate between the two t_G-values initially selected. Often a visual examination of the three chromatograms will remove any doubts concerning band assignment. Additionally, DryLab G with the original peak assignments can be used to predict retention times for the third run, and these can be compared with the values ac-

tually found. We will illustrate this using the two runs of Figure 8 as inputs to DryLab G, assuming both correct and incorrect band assignments. Resulting predictions of retention for an intermediate run (t_G = 60 min, Fig. 10) are compared in Table VI with experimental values. It can be seen in this example that bands #8/9 have reversed between the 30-min and 90-min runs.

Alternatively, band-areas can be converted into area-% values and compared between the two starting runs. This is also illustrated in Table VI for the bands in question. Again, the correct peak assignments are obvious when these data are examined.

Unresolved (missing) bands. In the case of unknown samples, it is not known how many bands should be seen when the sample is fully resolved. Therefore we cannot determine whether unresolved bands are present in a chromatogram just by counting peaks. This problem becomes more serious when small bands are overlapped by larger bands. In such cases, some small bands may be resolved in one run and unresolved in another, and resolved (but different) bands in each run may be mistakenly assumed to match each other. Problems of this type are usually best avoided by either (a) carrying out additional runs with t_G varying, and/or (b) increasing the space within the chromatogram so as to be able to accomodate a larger number of fully-resolved bands. That is, when values of N and $\bar{k}$ are increased (by increasing column length, flowrate or t_G, or by using smaller-particle columns), it is less likely that unresolved bands will occur in any given run. Studies to be reported [33] on the separation of ribosomal proteins have shown that long columns (25 cm), low flowrates (0.7 mL/min) and shallow gradients (25–45% acetonitrile in 60 and 240 min) are required to properly apply computer simulation. This problem is further discussed in Ref. [5].

Retention-time errors. If values of t_g for the two input runs for computer simulation are in error, errors in the resulting predictions by computer simulation can be even larger. Usually retention times can be measured experimentally with adequate precision. However when two bands are partly resolved, errors in the resulting values of t_g can occur (see discussion of Ref. [1], pp. 43–44). This problem is especially critical when the bands in question exhibit only minor changes in band-spacing as t_G is varied. In these cases, predicted resolution values can have a large relative error. This problem can also be mitigated by increasing the peak capacity of the system (longer column, lower flowrate, etc.). If doubt exists as to the reliability of starting t_G-values, additional experimental runs should be carried out and used for independent simulations. Simulated separations based on the two different sets of starting experimental runs should agree with each other, for prediction of a given separation.

Errors in the gradient. DryLab G assumes that the HPLC equipment is capable of providing gradients which are in close agreement with the desired shape. Computer simulation also requires an accurate measurement of the gradient dwell volume V_D. Both questions can be resolved by measuring the gradient shape, as described in Ref. [18]. A UV-absorber is added to the second solvent of the gradient system, so that a tracing of the gradient shape results when a gradient is run without sample injection and with the column removed.

Table V. Potential problems in the use of computer simulation for predicting gradient separation

Problem	Remedy
1. mismatched bands between two chromatograms	run additional run(s) with different t_g; verify that retention times are correctly predicted; measure %-areas for various bands to confirm peak matching
2. unresolved bands in complex samples (unknown)	run additional runs with different t_g; verify that number of bands in each chromatogram is correctly predicted, along with their retention times increase peak capacity by using longer, more efficient columns, slower flowrates, and/or longer gradient times
3. errors in retention time for poorly resolved bands	run additional runs with different t_g; confirm that same resolution vs t_g is predicted using different run-pairs with DryLab G increase peak capacity as above (#2)
4. errors in experimental gradients or estimate of dwell volume	check gradient shape by running analog gradient avoid early and late-eluting bands in initial (experimental) gradient runs by using broad gradient-range predictions for segmented gradients may be less accurate
5. curved plots of log k' vs ϕ	less important as a contribution to error in predicted results for R_S; t_g-values may be less accurate for extrapolated simulations

Table VI. Matching bands between two runs used as input for computer simulation. Bands #6–11 of herbicide sample of Figs. 8 and 10.

Band position*	area-% t_g = 30	area-% t_g = 90	retention for t_g = 60 min expt.	simulated correct**	simulated incorrect**
6	3.6	4.0	24.4	24.6	24.6
7	5.1	4.5	25.9	26.0	26.0
8	4.8	11.9	27.0***	27.2	27.0
9	12.2	5.0		27.2	27.3
10	5.0	5.1	27.8	27.9	27.9
11	8.8	9.1	28.5	28.6	28.6

* elution order in each run

** the correct assignment: band #8 (t_g = 30) is the same as band #9 (t_g = 90); the incorrect assignment assumes that peak order is the same in the two runs

*** peaks #8 and 9 fuse in the actual 60-min run; this is predicted by computer simulation with the correct assignments of bands in the two starting runs; the incorrect assignment predicts R_S = 0.9 for bands #8/9 in the 60-min run (cf. Fig. 10)

Curvature in plots of log k′ vs φ. Eq. (3) is often not an exact description of experimental plots of k′ vs φ, although resulting errors are of marginal practical importance [18]. It is possible to correct for these effects as described in Ref. [18]. These corrections will be incorporated into the DryLab G software shortly, but the present examples are not corrected.

Conclusions

Computer simulation provides an alternative to trial-and-error experimental runs for the purpose of improving separations by gradient elution. A simulated run can be carried out in less than a minute, saving more than 90% of the time required for an actual gradient-elution separation. At the same time the computer can present the results of many possible experiments as convenient plots of sample resolution vs gradient time (relative-resolution map). A computer can also facilitate systematic method development by holding relative retention fixed while column conditions (column length and diameter, particle size and flowrate) are varied for further improvement of separation. These various factors can result in the faster development of better methods, even by chromatographers having little experience with gradient elution.

The present study has analyzed the performance of commercial software (DryLab G) for carrying out computer simulations for reversed-phase gradient elution. Two experimental gradient separations of the sample of interest are run initially, followed by computer simulation. For several different samples, it is shown that retention predicted in this way agrees closely with experimental results. A systematic approach to method development for reversed-phase gradient elution is also presented, and its application to several samples is shown. This approach relies strongly on "solvent-strength optimization", based on band-spacing changes that result when gradient conditions are changed so as to change relative sample retention during elution through the column. Several examples of this effect can be seen in the present examples.

Acknowledgements

We are grateful to Dr. Roy Eksteen of Supelco., for giving us the column for the PAH separations. We also thank Drs. K. A. Cohen (Boehringer-Ingelheim) and J. D. Stuart (Univ. of Connecticut) for providing the raw data cited in Ref. [32].

Glossary of Terms

b	gradient steepness parameter (eq. 5)
b_i, b_j	values of b (for a given solute) in runs i and j differing only in t (eqs. 4a, b)
F	mobile-phase flowrate (mL/min)
G	gradient compression factor; see Ref. [1]
J	anomalous band-broadening factor in gradient elution; see Ref. [7]
k'	solute capacity factor; also instantaneous value in gradient elution at a given time (eq. 2)
$\bar{k}$	value of k' for a band in gradient elution when the band has migrated halfway through the column
k_f	value of k' for a band as it leaves the column during gradient elution (eq. 10)
k_0	value of k' for a given solute at the beginning of the gradient (for $\phi = \phi_0$)
k_w	value of k' for water as mobile phase (eq. 3)
N	plate number in corresponding isocratic separation where k' (isocratic) = $\bar{k}$ (gradient)
PAH	polyaromatic hydrocarbon
RRM	relative-resolution map (see Figs. 3, 6, 9)
R_s	resolution of two adjacent bands (eqs. 1, 11)
S	$d(\log k')/d\phi$ (eq. 3, isocratic elution)
SD	standard deviation
t	time (min) after sample injection and start of gradient
t_D	dwell time for a given HPLC system (min); equal to V_D/F
t_g	retention time (min) in gradient elution
t_G	gradient time (min)
$(t_g)_i$, $(t_g)_j$	values of t_g for a given solute in runs i and j, differing only in t_G (min)
t_0	column dead-time (min)
t_R	retention time (min) in isocratic elution
t_1, t_2	values of t_g for first and second (adjacent) bands (min)
V_D	dwell volume for a given HPLC system (mL)
V_m	column dead-volume (mL), equal to (t_0 F)
W	baseline bandwidth (min)
W_1, W_2	values of W for first and second (adjacent) bands (min)
α	separation factor (eq. 11)
ϕ	volume-fraction of organic solvent in mobile phase
ϕ_0	initial value of ϕ for a gradient
$\Delta\phi$	change in ϕ during the gradient

References

[1] *L. R. Snyder, J. J. Kirkland*, Introduction to Modern Liquid Chromatography, 2nd edn., Wiley-Interscience, New York 1979, Chap. 16.

[2] *L. R. Snyder*, in High-performance Liquid Chromatography. Advances and Perspectives, Vol. 1, *Cs. Horváth*, ed., Academic Press, New York, 1980, p. 207.

[3] *P. Jandera, J. Churacek*, Gradient Elution in Column Liquid Chromatography, Theory and Practice, Elsevier, Amsterdam, 1985.

[4] *L. R. Snyder, M. A. Stadalius*, in High-performance Liquid Chromatography. Advances and Perspectives, Vol. 4, *Cs. Horváth*, ed., Academic Press, New York, 1986, p. 195.

[5] *J. L. Glajch, M. A. Quarry, J. F. Vasta, L. R. Snyder*, Anal. Chem., **58**, 280 (1986).

[6] *P. Jandera, M. Spacek*, J. Chromatogr., **366**, 107 (1986).

[7] *M. Kunitani, D. Johnson, L. R. Snyder*, J. Chromatogr., **371**, 313 (1986).

[8] *J. W. Dolan, L. R. Snyder*, Chromatography Mag., **2**, 49 (1987).

[9] *M. A. Stadalius, H. S. Gold, L. R. Snyder*, J. Chromatogr., **327**, 27 (1985).

[10] *A. J. Banes, G. W. Link, L. R. Snyder*, J. Chromatogr., **326**, 419 (1985).

[11] *R. W. Stout, S. I. Sivakoff, R. D. Ricker, L. R. Snyder*, J. Chromatogr., **353**, 439 (1986).
[12] *Y. Baba, N. Yoza, S. Ohashi*, J. Chromatogr., **350**, 461 (1985).
[13] *S. Tomellini, S.-H. Hsu, R. A. Hartwick*, Anal. Chem., **58**, 904 (1986).
[13a] *Z. El Rassi, Y.-F. Maa, F. D. Antia, Cs. Horváth*, presented at the 10th Intern. Symp. on Col. Liq. Chromatography, San Francisco, May 18–23, 1986.
[14] *J. L. Glajch, JJ. Kirkland*, J. Chromatogr., **255**, 27 (1983).
[15] *J. L. Glajch, J. J. Kirkland*, J. Chromatogr. Sci., **25**, 4 (1987).
[16] *W. R. Melander, Cs. Horváth*, in High-performance Liquid Chromatography. Advances and Perspectives, Vol. 2, *Cs. Horváth*, ed., Academic Press, New York, 1980, p. 114.
[17] *L. R. Snyder, J. W. Dolan, J. R. Gant*, J. Chromatogr., **165**, 3 (1979).
[18] *L. R. Snyder, M. A. Quarry*, J. Liq. Chromatogr., in press.
[19] *M. A. Quarry, R. L. Grob, L. R. Snyder*, Anal. Chem., **58**, 907 (1986).
[20] *M. A. Quarry, R. L. Grob, L. R. Snyder*, J. Chromatogr., **285**, 1 (1984).
[21] *E. S. Parente, D. B. Wetlaufer*, J. Chromatogr., **355**, 29 (1986).
[22] *R. W. Stout, J. J. DeStefano, L. R. Snyder*, J. Chromatogr., **282**, 263 (1983).
[23] *L. R. Snyder, P. E. Antle*, LC, Liq. Chromatogr. HPLC Mag., **3**, 98 (1985).
[24] *M. A. Stadalius, M. A. Quarry, L. R. Snyder*, J. Chromatogr., **327**, 93 (1985).
[25] *L. R. Snyder, M. A. Stadalius, M. A. Quarry*, Anal. Chem., **55**, 1412A (1983).
[26] *P. Jandera, J. Chruacek, H. Colin*, J. Chromatogr., **214**, 35 (1981).
[27] *L. R. Snyder, J. W. Dolan*, LC/GC Mag., **4**, 921 (1986).
[28] *M. A. Quarry, R. L. Grob, L. R. Snyder, J. W. Dolan, M. Rigney*, J. Chromatogr., **384**, 163 (1987).
[29] *L. R. Snyder, M. A. Quarry, J. L. Glaijch*, Chromatographia, **24**, 33 (1987).
[30] *M. A. Stadalius, H. S. Gold, L. R. Snyder*, J. Chromatogr., **296**, 31 (1984).
[31] *M. A. Quarry, R. L. Grob, L. R. Snyder*, J. Chromatogr., **285**, 19 (1984).
[32] *M. Eslami, J. D. Stuart, K. A. Cohen*, J. Chromatogr., in press.
[33] *B. F. D. Ghrist, B. S. Cooperman, L. R. Snyder*, J. Chromatogr., to be submitted.

Received: June 29, 1987
Accepted: Sept. 9, 1987
F