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Application of the gradient elution technique

Demonstration with a special test mixture and the DryLab G/plus method development software

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ABSTRACT

A mixture of ten compounds with an overlapping peak pair was analysed with a 90-min elution gradient. To improve the separation, two reversed-phase gradients differing by a factor of 3 in their run times, were applied. Contrary to expectation, two peak pairs were less well separated in the gradient run with the lower slope. The relative resolution map provided a rapid solution to the problem: a gradient with 16-min run time gave the best separation of the mixture. The simulated chromatogram was verified experimentally. The differences between the predicted and experimental retention times averaged 0.03 min. Further improvement was obtained using a segmented gradient, which adequately separated all peaks in only 9 min.

INTRODUCTION

The use of gradient elution techniques is increasing in the quality control of pharmaceuticals [1]. The underlying model of the theory of gradient elution [2] has been used in computer simulations for the successful prediction of experimental results [3–6]. Predictions are reliable for small [7] and large molecules [8–10].

There are, however, several experimental problems encountered when the powerful technique of gradient elution is first used routinely. To reduce the difficulties in practical applications, we have developed a course in high-performance liquid chromatography (HPLC) using a special mixture for gradient elution which contains ten compounds of a broad range of polarity from acetone to pyrene. This course uses the method development software DryLab G/plus [11].

EXPERIMENTAL

The chromatograph used was a Hewlett-Packard 1090 M gradient system (Waldbronn, Germany). Water and methanol were degassed with helium prior to use. The detection wavelength was 254 nm. DryLab G/plus software (LC Resources, Orinda, CA, USA; Molnar, Berlin, Germany) was used with an MS-DOS-based computer system.

The chemicals and eluents (HPLC grade) were purchased from Fluka (Buchs, Switzerland). All HPLC parameters are given in Table I. The Nucleosil C_{18} column (125 × 4.6 mm, 5 μm) (Macherey and Nagel, Düren, Germany) was packed by the Central Analytical Department, Ciba Geigy (Basle, Switzerland). Experiments were carried out at 30°C.

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TABLE I

INPUT PARAMETERS FOR OPTIMIZATION OF THE MOBILE PHASE USING DRYLAB G/PLUS SOFTWARE

HPLC system		Gradient	
Column length (cm)	12.5	Start (%B)	5
Column I.D. (cm)	0.46	End (%B)	100
Flow-rate (ml/min)	1.0	Gradient time, run 1 (min)	20
Dwell volume[a]	0.35	Gradient time, run 2 (min)	60
Number of peaks	10		

[a] Dwell volume: *ca.* 2 ml for high-pressure mixer; *ca.* 5 ml for low-pressure mixer; *ca.* 0.3 ml for HP-1090.

RESULTS AND DISCUSSION

Sample

The computer-assisted HPLC method development procedure used here has been published previously [12] with several applications to various samples. For teaching the gradient elution technique to beginners, a special sample was mixed. With this test mixture various aspects of gradient elution in reversed-phase chromatography can be illustrated, such as the inversion of peak elution order, early or late eluting peaks and selectivity changes due to steps of different lengths and slopes.

Input data: two gradient runs

Two different gradient runs from 5 to 100% methanol in (a) 20 min (Fig. 1a) and (b) 60 min (Fig. 1b) were carried out using the sample mixture. The system parameters were entered into the DryLab G/plus software (Tables I and II). The measurement of the dwell volume was performed according to the DryLab G/plus instruction manual.

Isocratic conditions

It was not feasible to use isocratic method development for this sample with the DryLab I/plus software. The ratio of the capacity factor (k') values for the last and the first peaks was 1372, much larger than the value of 20 under which isocratic elution is advisable. The DryLab I/plus system recommended the use of gradient elution.

Peak tracking

After the two basic gradient elutions, including the integration reports (Table III), had been performed an attempt was made to correlate the corresponding peaks in the two chromatograms. If the same amount of sample was injected in both runs, it was possible to find the same peak areas or percentage peak areas for well assigned peaks [13].

The same total peak area can be seen in both runs: 30 032 (20-min elution) and 29 498 (60-min elution) peak area units. The difference is only 1.7%. The individual peak areas also have very similar values. A deviation is seen only for naphthalene and xylene. These two peaks overlap in the 60-min run (peak area 5387), but are separated in the 20-min run (peak areas 4326 and 1185). The sum of the areas of both peaks in the 20-min run is very close to the peak area of the overlapping peaks in the 60-min run (5511 *versus* 5387).

In some other instances deviations occur if the integration is not carried out correctly. It is then reasonable to check the position of the baseline and to interpret the integration results. The baseline in both the gradient runs reported here was excellent.

Critical resolution map

The map of critical resolution is shown in Fig. 2. It can immediately be seen where it is possible to separate all the peaks and where this is not possible. It is seen that there are two possible maxima for resolution at about 8 and 16 min. These two runs have the largest critical resolution between the bands for xylene and naphthalene at a gradient time of 13 min, where there is zero resolution between benzene and 4-hydroxybenzoic acid methyl ester. Using such a 13-min gradient (5–100% B), these two peaks completely overlap and appear as a single homogeneous peak.

The 8-min gradient with a slope of 12.5% B per min was considered too steep and was therefore neglected.

Simulation of several runs on the computer

The 16 min gradient run was simulated on the computer. The software generated a chromatogram on the monitor in a few seconds, in which the two cricital band pairs were well separated with nearly baseline resolution. A print-out of this simulated run is given in Fig. 3a.

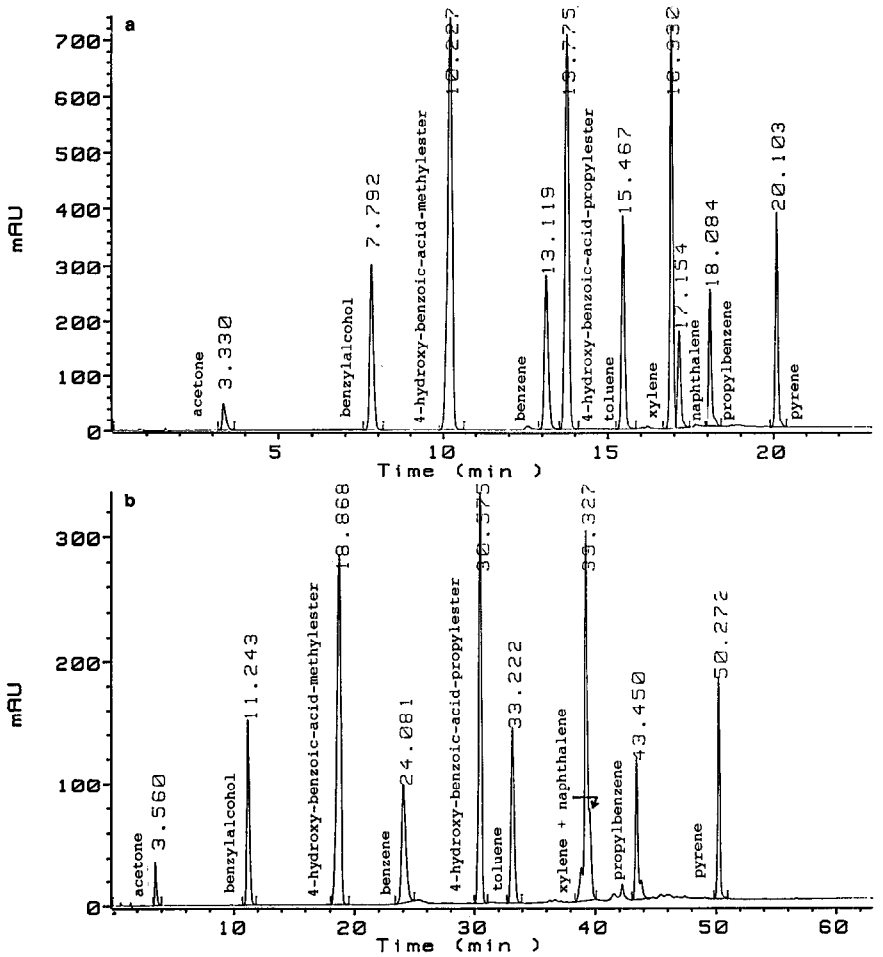

Fig. 1. (a) Basic gradient run No. 1 from 5 to 100% B in **20 min**. Chromatographic conditions as in Tables I and II. Detection wavelength, 254 nm; 1.2 a.u.f.s. (b) Basic gradient run No. 2 from 5 to 100% B in **60 min**.

TABLE II

PEAK PARAMETERS FOR OPTIMIZATION USING DRYLAB G/PLUS SOFTWARE

Peak no.	Compound	Retention time (min)		Peak area[a] (%)
		Run 1	Run 2	
1	Acetone	3.330	3.560	1.21
2	Benzylalcohol	7.792	11.243	7.73
3	4-OH-BA-ME[b]	10.227	18.868	24.93
4	Benzene	13.119	24.081	7.60
5	4-OH-BA-PE[c]	13.775	30.575	19.62
6	Toluene	15.467	33.222	8.61
7	Xylene	16.930	39.327	14.41
8	Naphthalene	17.154	39.600	3.95
9	Propylbenzene	18.084	43.450	4.87
10	Pyrene	20.103	50.272	7.07

[a] Peak area is taken from the reference run (here the 60-min run) and it is only for visualisation of the peaks, not for calculations.
[b] 4-OH-BA-ME = 4-hydroxybenzoic acid methyl ester.
[c] 4-OH-BA-PE = 4-hydroxybenzoic acid propyl ester.

TABLE III

PEAK TRACKING USING PEAK AREAS

60 min		20 min		Area ratio 60/20
Retention time (min)	Area	Retention time (min)	Area	
3.553	356	3.330	1364	0.97
11.241	2315	7.792	2321	0.99
18.868	6773	10.227	7487	0.90
24.080	2412	13.119	2283	1.06
30.575	5602	13.775	5892	0.95
33.222	2702	15.467	2587	1.04
38.920	457	16.930	4326	0.10
39.327	5387	17.154	1185	4.54
43.459	1320	18.084	1462	0.90
50.272	2174	20.103	2125	1.02
Total	29 498		30 032	0.98

Correlation between computer simulation and experimental results

The experimental control of the chromatogram suggested by the software revealed that the correlation between the predicted retention times and those found experimentally, as shown previously [10–14], was fairly good (Fig. 3b).

The average deviation in the retention times was less than 0.03 min (<2 s) (Table IV).

Up to now, participants of the gradient-HPLC-course did not find this simple optimum without the help of DryLab. Their solution was usually a multi-segmented gradient of 20–30 min, with a tendency to longer and longer runs, searching for improved resolution.

A systematic search for an optimum one-step linear gradient is shown in Fig. 4 (Table V). Fixing the content of B at the end of the gradient to 100% and changing the content of B at the start from 10 to 60 in steps of 5%, data were obtained for the critical resolution. It can be seen that gradients starting from 40, 45 and 50% B are not robust. Also gra-

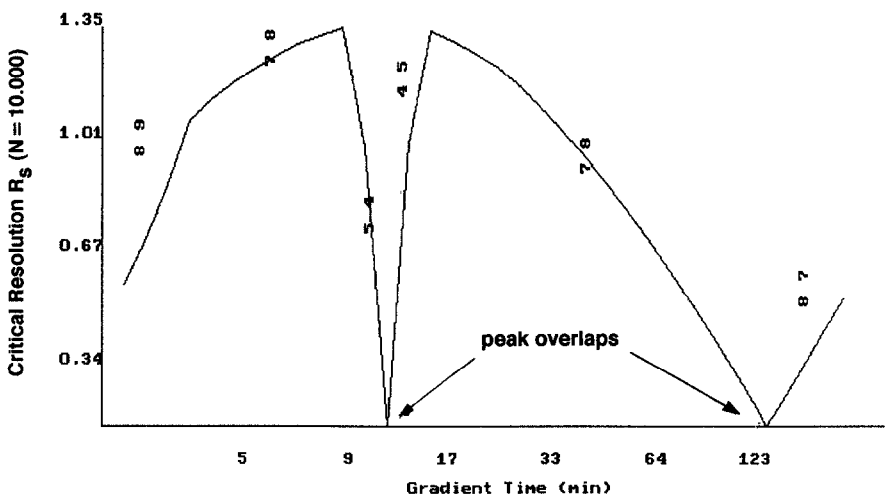

Fig. 2. Critical resolution map showing the lowest (critical) resolution between the two worst separated bands (numbers above the curve) as a function of the gradient time. Highest resolution is observed at 8- and at 16-min gradient run time (5–100% B). All other conditions as in Fig. 1.

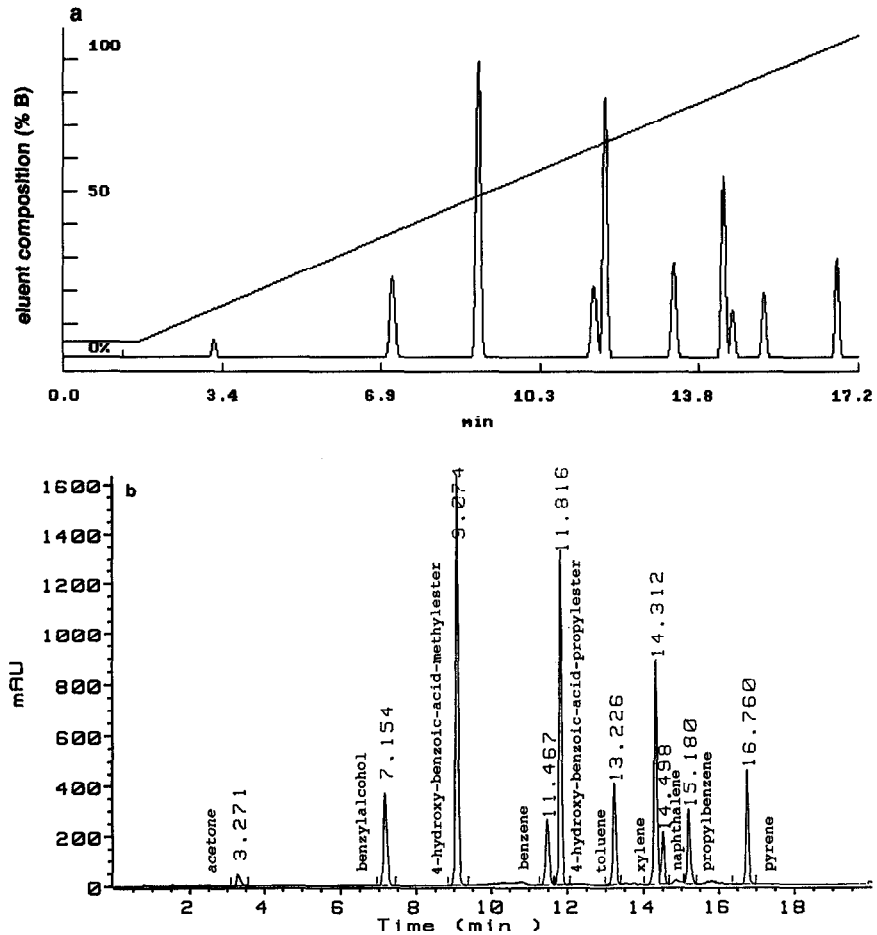

Fig. 3. (a) Predicted 16-min gradient run (5–100% B) by DryLab G/plus software. (b) Experimental 16-min run (5–100% B) (other conditions as in Fig. 1).

TABLE IV

COMPARISON OF RETENTION TIMES PREDICTED BY DRYLAB G/PLUS AND THOSE FOUND EXPERIMENTALLY

Peak no.	Compound	Retention time (min)		Difference in retention time (min)
		Predicted	Experimental	
1	Acetone	3.26	3.27	0.01
2	Benzylalcohol	7.13	7.15	0.02
3	4-OH-BA-ME[a]	8.99	9.07	0.08
4	Benzene	11.51	11.47	0.04
5	4-OH-BA-PE[b]	11.75	11.82	0.07
6	Toluene	13.24	13.23	0.01
7	Xylene	14.32	14.31	0.01
8	Naphthalene	14.52	14.50	0.01
9	Propylbenzene	15.20	15.18	0.02
10	Pyrene	16.78	16.76	0.02

[a] 4-OH-BA-ME = 4-hydroxybenzoic acid methyl ester.
[b] 4-OH-BA-PE = 4-hydroxybenzoic acid propyl ester.

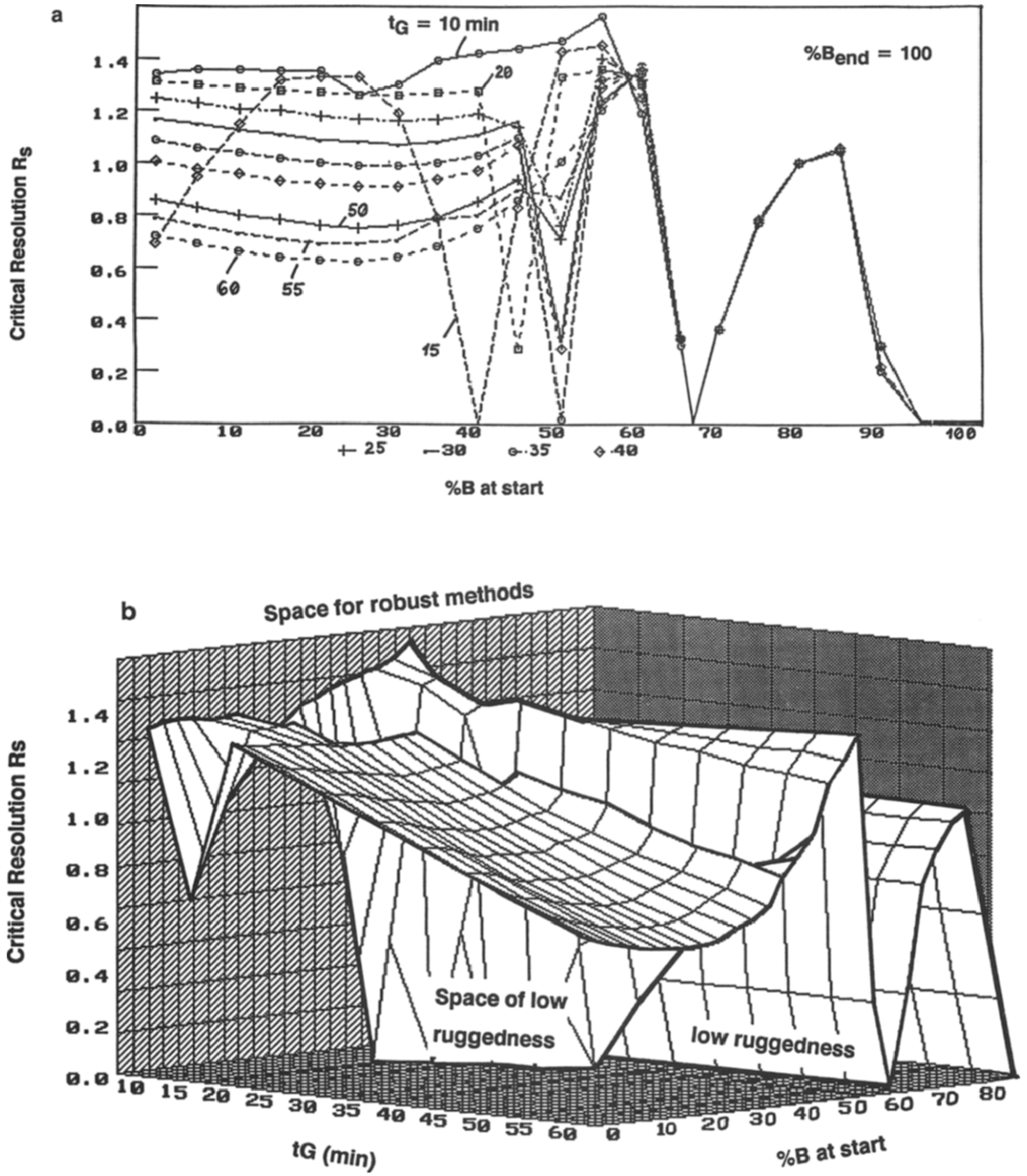

Fig. 4. (a) Multiple relative resolution map for the sample using a one-step linear gradient from a varying percentage of B at the start to a fixed percentage (100%) of B at the end of the run. There are several valleys in this graph, which means that the methods are of low ruggedness. A maximum is seen around a gradient time of 10 min and a percentage of B at the start of 5%. (b) Three-dimensional plot of relative resolution maps as described in (a).

TABLE V

CRITICAL RESOLUTION VALUES USING A FIXED PERCENTAGE OF B AT THE END (100%) AND A VARIABLE PERCENTAGE OF B AT THE START OF THE ELUTION

Elution time varied from 10 to 60 min.

Percentage of B at start	Gradient time (min)										
	10	15	20	25	30	35	40	45	50	55	60
0	1.34	0.69	1.31	1.25	1.17	1.09	1.01	0.93	0.86	0.79	0.72
	1.36	0.95	1.30	1.23	1.15	1.06	0.98	0.90	0.83	0.76	0.69
10	1.36	1.15	1.29	1.21	1.13	1.04	0.96	0.88	0.80	0.73	0.66
	1.35	1.32	1.28	1.20	1.11	1.02	0.93	0.85	0.78	0.71	0.64
20	1.35	1.33	1.27	1.18	1.09	1.00	0.92	0.84	0.76	0.69	0.63
	1.26	1.33	1.26	1.17	1.08	0.99	0.91	0.83	0.75	0.69	0.62
30	1.30	1.19	1.26	1.16	1.07	0.99	0.91	0.83	0.76	0.70	0.64
	1.39	0.79	1.27	1.17	1.08	1.00	0.94	0.86	0.79	0.78	0.68
40	1.42	0.00	1.28	1.19	1.11	1.03	0.97	0.90	0.85	0.80	0.75
	1.44	0.83	0.29	1.14	1.16	1.10	1.07	0.99	0.94	0.90	0.86
50	1.47	1.43	1.33	0.76	0.32	0.02	0.29	0.52	0.71	0.87	1.01
	1.56	1.45	1.36	1.40	1.36	1.32	1.29	1.27	1.24	1.22	1.20
60	1.19	1.25	1.30	1.32	1.33	1.34	1.35	1.36	1.36	1.37	1.37
	0.30	0.32	0.33	0.33	0.33	0.34	0.34	0.34	0.34	0.34	0.34
70	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36	0.36
	0.78	0.78	0.77	0.77	0.77	0.77	0.77	0.77	0.77	0.77	0.77
80	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	1.06	1.06	1.05	1.05	1.05	1.05	1.05	1.05	1.05	1.05	1.05
90	0.20	0.22	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30
	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
100	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

dients starting with a content of B higher than 60% are not reasonable, as there is a resolution valley at 68% B. Between 68 and 92% B there is an improvement in resolution up to 1.06, but this is lower than other possible values in this plot.

A relatively robust area is between 55 and 60% B. It was therefore decided to search for a multistep gradient with a higher resolution and faster analysis time in this concentration region.

Multisegmented gradients for faster gradient runs (trial and error)

The software has the advantage of being able to simulate gradients with up to ten steps in any combination. In developing a multisegmented gradient, the goal was to reach a nearly equal band spacing of all bands. This is possible if all the components are at the largest possible distance from each other. This situation is characterized by the highest relative resolution between all bands and by the lowest standard deviation of all resolution (R_s) values for all band pairs.

To begin the optimization procedure, the chromatogram was divided into four parts. A 2-min isocratic step at 50% B was taken to give a large R_s value between bands 2 and 3. As the distance between bands 3 and 4 was large (2.5 min) (Fig. 3b), this distance was shortened by having a sharp step up to 77% B in 0.1 min; the distance between bands 2 and 3 could therefore be reduced to 0.15 min. This step was followed by another isocratic step at 77% B for 2.9 min to resolve bands 4 and 5 ($R_s = 5.08$) and also the critical peak pair 7 and 8 ($R_s = 1.55$). Finally, the content of B was increased from 77 to 100% in 3 min to give a fast elution of the late bands. The total analysis time should be less than 10 min (Fig. 5a).

Using the experimental control, a fairly good correlation was found with the DryLab prediction (Fig. 5b) (Table VI). The deviations are slightly

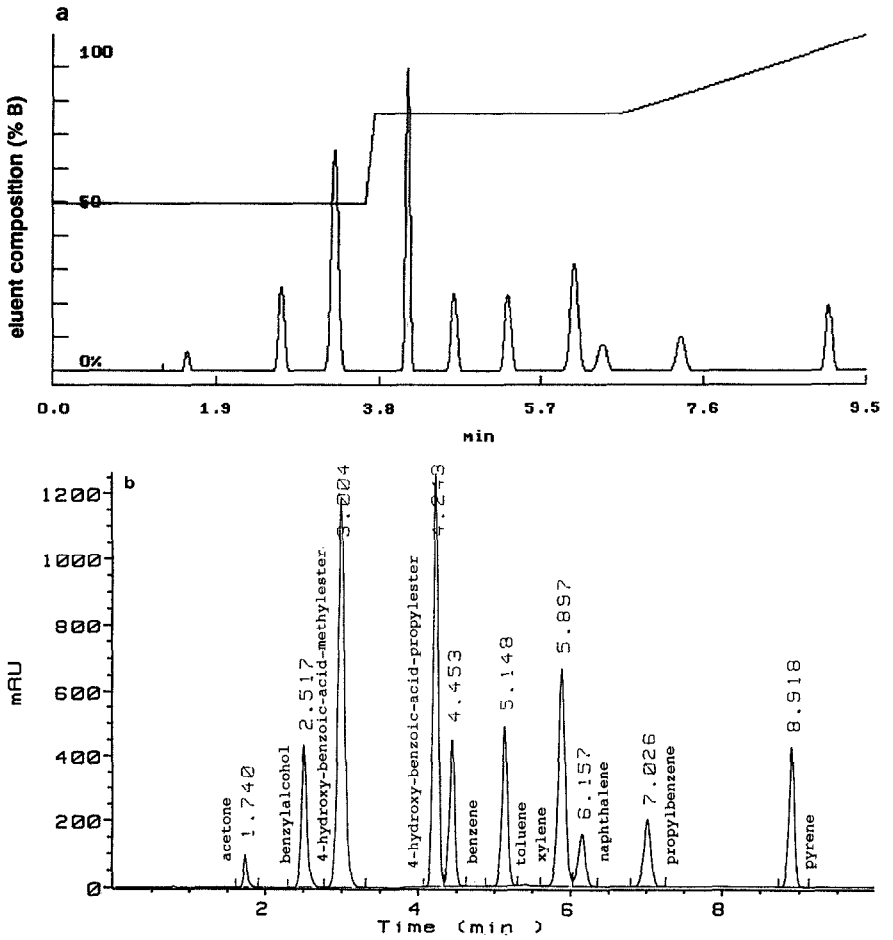

Fig. 5. (a) Predicted multisegmented gradient. Conditions for the steps are given in the text; the %B values were measured in the detector cell. (b) Experimental multisegmented gradient (conditions as in Fig. 1).

TABLE VI

COMPARISON OF RETENTION TIMES PREDICTED BY THE DRYLAB G/PLUS SOFTWARE AND THOSE FOUND EXPERIMENTALLY

Peak no.	Compound	Retention time (min)		Difference in retention time (min)
		Predicted	Experimental	
1	Acetone	1.56	1.74	0.18
2	Benzylalcohol	2.67	2.52	0.15
3	4-OH-BA-ME[a]	3.27	3.00	0.27
4	Benzene	4.65	4.45	−0.20
5	4-OH-BA-PE[b]	4.11	4.24	0.13
6	Toluene	5.30	5.15	−0.15
7	Xylene	6.09	5.90	−0.19
8	Naphthalene	6.43	6.16	−0.27
9	Propylbenzene	7.33	7.03	−0.30
10	Pyrene	9.05	8.92	−0.13

[a] 4-OH-BA-ME = 4-hydroxybenzoic acid methyl ester.
[b] 4-OH-BA-PE = 4-hydroxybenzoic acid propyl ester.

larger than in Table IV, which is probably a result of the large step at the beginning of the run (Fig. 5a) being too harsh for the phase system. The step is actually rounded, which explains the slight deviations.

The predicted inversion of the elution order for benzene and 4-hydroxybenzoic acid propyl ester was also seen in this chromatogram.

Another method of studying the elution order is using the S values, which are the slopes of $\ln k' = f(\%B)$ and which are calculated by the software together with the $\log k'_w$ values. Peak cross-overs can often be recovered on the basis of the slopes of the individual compounds.

The DryLab G/plus software can be an efficient tool in teaching gradient elution to those who are not familiar with the technique and who do not have the time to run a large number of trial-and-error experiment. A more robust and less time-consuming method could be developed by shortening the gradient. Pollution is also reduced by the reduction of organic eluent wastes.

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