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Simultaneous variation of temperature and gradient steepness for reversed-phase high-performance liquid chromatography method development
II. The use of further changes in conditions

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Abstract

The preceding paper (Part I) suggests that simply optimizing temperature and gradient steepness will often provide an adequate reversed-phase HPLC separation. In some cases, however, this procedure will prove unsuccessful, and then further method-development experiments (involving change in other separation conditions) will be required. One strategy is to change a variable other than temperature or gradient steepness, followed by re-optimization of the latter two variables. The present paper examines the application of this approach with the aid of computer simulation to several samples. © 1998 Elsevier Science B.V.

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1. Introduction

An optimal strategy for reversed-phase high-performance liquid chromatography (HPLC) method development should recognize several possible goals:

1. Achievement of adequate sample resolution; e.g., baseline separation ($R_s > 1.5$) for all bands.
2. Completion of method development with the fewest number of experimental runs and the least effort (preferably with the help of appropriate method-development software).
3. A final HPLC method which is rugged and convenient to carry out, with a run-time that is as short as possible.
4. A method-development procedure which is applicable to as wide a range of sample types as possible; e.g., neutrals and ionics, samples that require low-UV detection, etc.
5. A method-development procedure that results in a high percentage of successful separations.

A general method-development strategy [1] is to begin with acetonitrile–water as the mobile phase, and adjust retention so that $0.5 < k < 20$ for every sample component in isocratic separation (or $0.5 < k^* < 20$ in gradient elution). If further improvement of separation is needed, band spacing (i.e., selectivity

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or values of α) can be varied by changing any of several separation conditions. Finally, column conditions (column length, particle size and flow-rate) can be changed, either for increased resolution or reduced run-time. Where most HPLC method development procedures differ is in the choice of variables for optimizing band spacing [2].

It has been suggested previously [3–5] that a good starting point for optimizing band spacing is to vary temperature T and gradient steepness (or gradient time t_G). The preceding paper (Part I [6]) has presented some examples of this approach for selected samples, while taking advantage of computer simulation to minimize the required number of method-development experiments. Although these preliminary results are promising, they also suggest that some samples cannot be separated just by optimizing T and t_G. In these cases, one option is to change one or more additional separation conditions, followed by re-optimization of T and t_G, as a means of further improving separation. This approach to method development is examined in the present paper for different variables and samples.

2. Experimental

Equipment, materials and procedures are described in Part I [6], except for additional details given in the text and/or figure captions.

3. Results and discussion

When faced with inadequate resolution following the optimization of T and t_G, there are several options: (i) for a modest change in resolution (up to a 1.5-fold increase in R_s), an increase in column length combined with a decrease in flow-rate can be effective, at the expense of a longer run-time; (ii) smaller increases in resolution for gradient separations can sometimes be achieved by optimizing initial % B for the gradient, and/or the use of segmented gradients (where gradient steepness is optimized for different parts of the chromatogram) [1,7], following which T and t_G can be re-optimized and (iii) for larger changes in resolution, a variable (other than T or t_G) can be changed that is known to have a potentially large effect on band spacing (e.g., pH, solvent or column type, ion-pairing, etc.), following which T and t_G can be re-optimized.

Each of these three options will be examined.

3.1. Change in column conditions

A change in column length, particle diameter or flow-rate is referred to as a change of ''column conditions'' [1]. Often the initial separation will be carried out at a pressure that is close to the maximum desirable pressure (e.g., 2000 p.s.i.; 1 p.s.i.=6894.76 Pa), and any change in column conditions should not lead to an unacceptably high pressure. Therefore, if column length is increased, it may be desirable to decrease flow-rate in the same proportion, so as to maintain pressure constant. If column length is doubled (by adding a second equivalent column in series) and flow-rate decreased by half, resolution will usually increase by 50% and run-time will increase 4-fold. Thus, the primary cost of increasing resolution in this way is an increase in run-time.

Table 1 summarizes the use of a change in column conditions to improve the separation of selected samples described in Part I (Table 2 of Ref. [6]), following optimization of T and t_G for these

Table 1
Use of a change in column conditions to improve resolution or reduce run time for selected samples of Part I (Table 1 of Ref. [6])

Sample	Initial conditions, R_s and run time[a]					Final conditions, R_s and run time[a]				
	L	F	d_p	R_s	Time	L	F	d_p	R_s	Time
(2) Synthetic organics	12.5	1.0	5.0	2.4	26	5.0	2.5	5.0	1.6	5
(5) Fatty acid esters	25	1.0	5.0	0.3	25	50	0.5	5.0	0.5	88
(11) LSD derivatives	25.0	1.0	5.0	0.8	14	50	0.5	5.0	1.2	56

Column pressure drop is held constant; computer simulation used to predict new values of R_s and run time.
[a] Column length L (cm), flow-rate F (ml/min), particle size d_p (μm); run time (min) is for elution of last peak (at which point the gradient should be terminated).

samples. In the case of sample 2 (synthetic organics), the initial resolution (R_s=2.4) is greater than required. By reducing column length by 2.5-fold (from 12.5 to 5 cm) and increasing flow-rate by the same factor (from 1 to 2.5 ml/min), run-time can be reduced from 26 to 5 min, while resolution is reduced from R_s=2.4 to 1.6 (baseline separation). For sample 11 (LSD derivatives) the initial resolution is too low (R_s=0.8). By doubling column length (25 to 50 cm) and reducing flow-rate by the same factor (1.0 to 0.5 ml/min), resolution can be increased to R_s=1.2 (marginal) while run-time is increased from 14 to 56 min. Finally, for sample 5 (fatty acid methyl esters), the initial resolution of R_s=0.3 must be increased. A similar doubling of column length (from 25 to 50 cm) and halving of flow-rate (1.0 to 0.5 ml/min) increases resolution to R_s=0.5, which is still unacceptable (complete band overlap for the critical pair 10/11). In each of these examples, gradient time must be changed so as to maintain (t_G F/L) constant, so as to preserve the band spacing that results from optimizing T and t_G [1], but see the important qualification of Ref. [8] when the equipment hold-up volume V_D is significant. For the latter case (large V_D), maintaining (t_G F/L) constant does not always ensure constant selectivity and band spacing, and Ref. [8] should be consulted for means to anticipate and avoid this problem.

3.2. Change in gradient conditions

3.2.1. Initial % B

The resolution of some samples can be improved by optimizing the initial % B value for the gradient. This can be accomplished conveniently by using the present computer simulation software; an automatic ''optimum linear gradient'' feature is available to the user. When this option is selected, the computer examines all possible combinations of initial % B, temperature and gradient time and selects conditions for maximum resolution. Conditions which result in the elution of the first band with a retention time less than $1.5t_0$ (k<0.5) are not allowed, since such separations are undesirable [1]. After the search for optimum conditions is completed, the computer trims the gradient for any wasted time at the end of the gradient. For increments in initial % B of 2% B or greater, calculation times for the use of this optimum linear-gradient feature are usually <1 min.

Table 2 summarizes the application of the optimum linear-gradient option (optimized T, t_G and initial % B) to the 14 samples described in Ref. [6]. For about half of these samples (Nos. 3, 4, 6, 8, 9,

Table 2
Optimized conditions (gradient and temperature) for the samples of Table 1 of Ref. [6]

No.	Sample	Gradient range (% B)		Conditions[a]		Maximum R_s[b]	
		Original	Optimized[c]	t_G (min)	T (°C)	Orig.	Opt.
1	Herbicides	20–95	6–53	42	34	1.6	1.7
2	Synthetic organic mixture	10–100	28–56	24	36	2.4	2.7
3	Acrylate monomers	0–100	0–86	74	18	1.4	1.4
4	Algal pigments	0–100	7–90	74	59	0.7	0.7
5	Fatty acid methyl esters	60–100	85–90	18	64	0.3	0.4
6	Corticosteroids	5–100	40–49	14	28	1.1	1.1
7	Testosterones	0–80	38–51	9	28	0.2	0.5
8	Herbicide impurities	5–95	12–42	9	38	4.7	4.7
9	Pharmaceutical compounds	20–100	22–71	95	44	6.0	6.1
10	Toxicology standards	0–100	12–79	33	30	0.7	0.9
11	Anilines	5–65[d]					
12	Benzoic acids	5–50	14–21	13	66	1.5	1.9
13	LSD derivatives	5–80	20–88	56	56	0.8	0.9
14	Protein digest	0–60	0–39	83	66	3.0	3.0

[a] For optimized gradient; e.g., 0–58% B for sample 1.
[b] ''Original'' values for original gradient and optimized T and t_G; ''new'' values for optimized gradient time and T for new gradient.
[c] Gradient automatically optimized by computer.
[d] Due to pre-elution of early bands, accurate predictions for a change in initial % B are not possible.

14), there is no improvement in resolution when initial % B is optimized. For the remaining samples (Nos. 1, 2, 5, 7, 10, 12, 13, except No. 11 – see footnote in Table 2), the average increase in resolution is 0.2 units, or 15%. This represents a small but significant advantage for simultaneously optimizing initial % B, T and t_G, especially since the run-time is usually shortened at the same time. The results of Table 2 are probably typical of what can be expected for other samples.

3.2.2. Segmented gradients

Segmented gradients can improve resolution when there are two critical pairs for optimized conditions of T and t_G, and these two band-pairs are situated in different parts of the chromatogram [1,7]. If the first band-pair (i/j) is better resolved with a less-steep gradient, while the second pair (p/q) prefers a steeper gradient (often the case), then a segmented gradient which provides the preferred gradient steepness for each band-pair will yield better resolution. A similar situation results when bands i/j prefer a steeper gradient and bands p/q prefer a less steep gradient (which is equally likely).

For even a modest increase in resolution from the use of segmented gradients, the band-pairs i/j and p/q must be well separated in the chromatogram, ideally eluting in mobile phase that differs by 50% B for the two band-pairs (see Appendix A). In most cases, the critical band-pairs i/j and p/q will be closer together in the chromatogram, and the increase in sample resolution from the use of a segmented gradient will be correspondingly less. As an example, consider sample 1 of Table 2 of the preceding paper (Part I [6]). For optimized values of temperature (34°C), gradient time (91 min), and the initial, non-optimized gradient range (20–95% B), the resolution of this herbicide impurities sample is R_s=1.56. An optimized segmented gradient can be found readily (using computer simulation) by trial-and-error changes in gradient conditions: 20/33/57% B in 0/22/23 min, with R_s=1.66. This improvement in resolution (0.1 unit) is barely significant (and similar to that obtained by optimizing initial % B for a linear gradient; Table 2); this small increase in resolution can be related to the fact that the two critical band-pairs within the chromatogram are separated by only 6% B. For the other samples of Table 2, the separation of the critical band-pairs varies from 0 to 6% B, and only minor improvements in resolution can be expected from the use of segmented gradients.

The optimization of a linear gradient, including initial % B, is generally preferable to the use of segmented gradients. Segmented gradients are subject to gradient ''rounding'' effects or dispersion of the gradient by the HPLC system [9]. As a result, computer predictions for segmented gradients are expected to be less reliable, and methods based on segmented gradients tend to be less transferable because of differences in gradient equipment. These potential problems with segmented gradients increase with the number of segments in the gradient, suggesting that multi-segment gradients should be avoided if rugged, transferable methods are required.

3.3. Change in other variables

A number of separation conditions can be changed in order to vary band spacing [1]. For the separation of ionic samples (mixtures of acids and/or bases), a change in pH or the use of ion-pairing can provide large changes in band spacing. For both neutral and ionic samples, the following variables can also affect band spacing: temperature (smallest effect)<% B or gradient steepness≈column type (e.g., C_{18}, phenyl, cyano)<solvent type (acetonitrile, methanol, tetrahydrofuran) (largest effect).

A previous study [3] has determined the relative ranking of these variables, as indicated above; i.e., a change in temperature is predicted to affect band spacing least, while a change in solvent type (e.g., substitution of methanol or tetrahydrofuran for acetonitrile) will have the greatest effect. When the simultaneous optimization of temperature, gradient time and one or more other separation conditions is attempted, a relatively large number of experiments is required. An alternative, when the initial optimization of T and t_G proves ineffective, is to change some third variable (e.g., column type) *once*, and then repeat the optimization of T and t_G (requiring an additional four experimental runs. The latter approach has been investigated for several of the samples from Part I [6], as described below.

3.4. Change of solvent type

A change of solvent type is expected to have a large effect on band spacing and should be a good choice when the initial optimization of T and t_G has not resulted in acceptable sample resolution. For practical reasons, it is preferable to avoid the use of tetrahydrofuran [1], which leaves acetonitrile and methanol as preferred solvents.

3.4.1. Acrylate monomers

This sample of Table 2 of Ref. [6] was separated with $R_s=1.38$ after T and t_G were optimized. The mobile phase in Ref. [1] was acetonitrile–water, so a change to methanol–water was anticipated to provide a further change in band spacing, with possibly improved resolution after re-optimizing T and t_G. The four experiments required to optimize T and t_G were: 0–100% methanol in water gradients in 20 and 60 min, at temperatures of 23 and 70°C. The same column (30×0.39 cm Novapak C_{18}) was used as in Ref. [1], with a flow-rate of 1.25 ml/min. Fig. 1b shows the resulting resolution map for methanol as solvent, for comparison with the acetonitrile resolution map in Fig. 1a. There are substantial differences in the two maps, but maximum resolution ($R_s=1.4$) is the same in both cases (cursor in Fig. 1a Fig. 1b indicates optimum T and t_G). As seen in the corresponding chromatograms of Fig. 1c Fig. 1d for conditions of maximum resolution, the band spacing is also quite similar for each separation. That is, there is not much change in selectivity as a result of changing the solvent. This is probably due to the chemical similarity of these predominantly acrylate-ester sample compounds, which also hinders changes in band spacing as a result of change in T or t_G [3].

3.4.2. Fatty acid methyl esters

This sample of Table 2 of Ref. [6] was separated with $R_s=0.32$ after T and t_G were optimized, using a monomeric C_{18} column. A resolution map for these conditions is shown in Fig. 2a, and the optimized separation is shown in Fig. 3a. The initial mobile phase was 60–100% acetonitrile in water (Fig. 2a, Fig. 3a), so a change to methanol–water was explored (using input data reported in Ref. [10]). The four experiments required to optimize T and t_G were: 75–100% methanol in water gradients in 30 and 90 min, at temperatures of 23 and 70°C. The same column (25×0.46 cm Zorbax SB-C_{18}) was used as in Ref. [1], with a flow-rate of 1.0 ml/min. For these latter conditions, Fig. 2b shows the resulting resolution map, which suggests optimum values of T (28°C) and t_G (154 min), for which $R_s=0.61$ (Fig. 3b). In this case, a change in solvent type resulted in a significant (2-fold) increase in resolution. However, separation is still marginal.

3.5. Change of column type

For most samples, a change from a C_8 or C_{18} column to a phenyl or cyano column will result in significant changes in band spacing [10]. Changes in column type are not expected to have as large an effect on band spacing as a change in solvent type, and such changes as occur tend to mimic those that result from a change in % B or gradient time [10]. For these reasons, a change in column type is usually not our first choice for a change in variable to create changes in band spacing. There are two important exceptions, however. First, isomeric compounds are often difficult to separate by means of reversed-phase HPLC, and samples containing isomers are likely to be better separated either by normal-phase HPLC (i.e., using a different column) or by reversed-phase HPLC with a cyclodextrin bonded-phase column (Ref. [1], pp. 250–251, 731–733). Second, compounds differing in molecular shape are often better separated either with alkyl-silica column packings that are ''polymeric'' (i.e., made from polychlorosilanes, vs. ''monomeric'' packings made from dimethylchloroalkylsilanes, [11]) or which have a longer alkyl chain (e.g., C_{30} [12]).

3.5.1. Fatty acid methyl esters

The separation of this sample by optimizing T and t_G for mobile phases of either acetonitrile–water or methanol–water is discussed above; a maximum resolution of $R_s=0.61$ was possible with a methanol–water gradient and a ''monomeric'' column (Fig. 2a, Fig. 3a). The molecular shapes of the present *cis*-olefinic fatty acid esters differ from each other, and for this reason a change in column type to a ''polymeric'' C_{18} packing was investigated next. Four experiments were carried out in order to optimize T and t_G: 60–100% acetonitrile in water

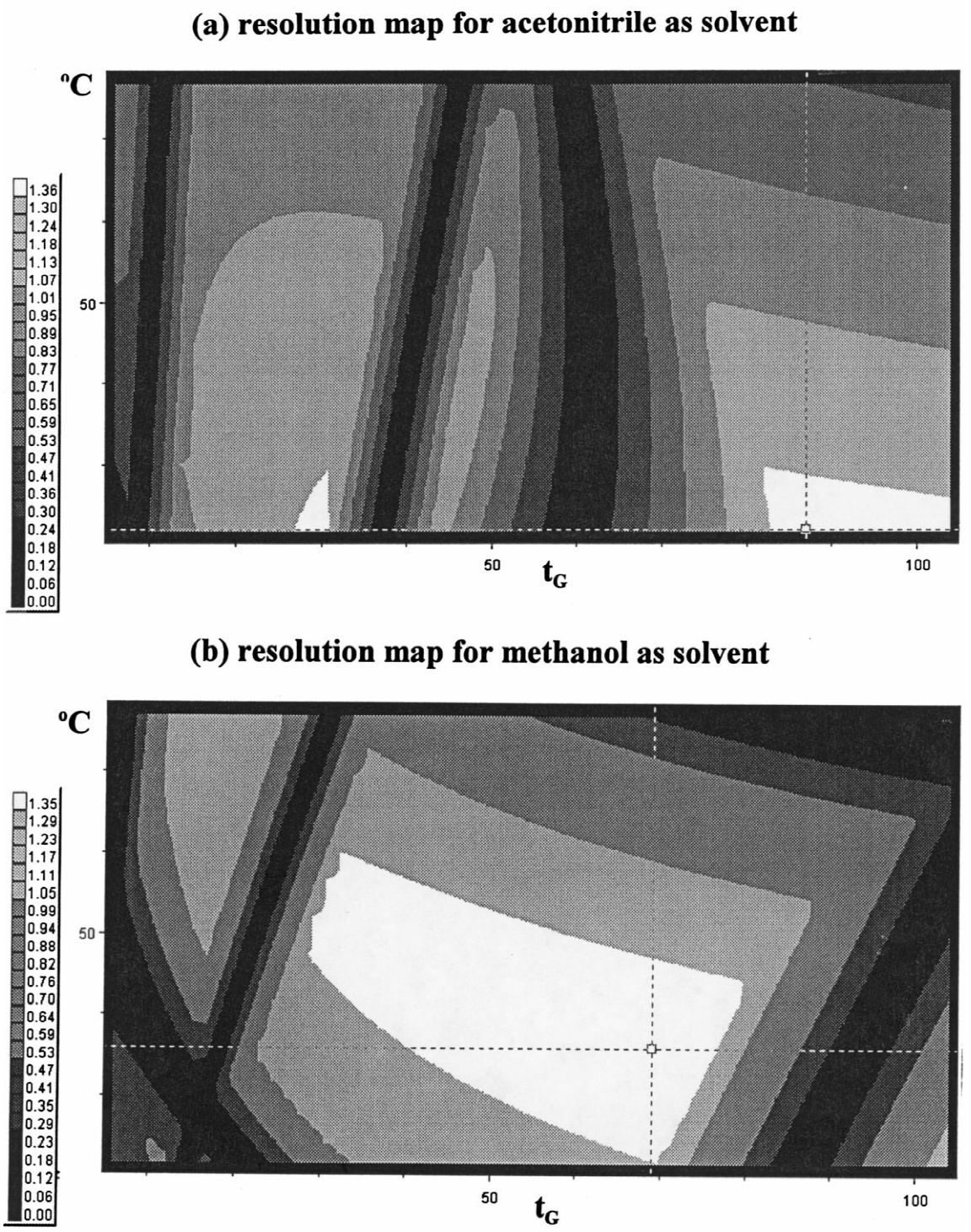


Fig. 1. Separation of acrylate monomer sample (No. 3). Conditions: 30×0.39 cm C_{18} column, 0–100% B gradients, 2.0 ml/min. (a) Resolution map for acetonitrile as solvent; (c) maximum-resolution separation from (a); 22°C, 84-min gradient; (b) resolution map for methanol as solvent; (d) maximum-resolution separation from (b); 34°C, 57-min gradient.

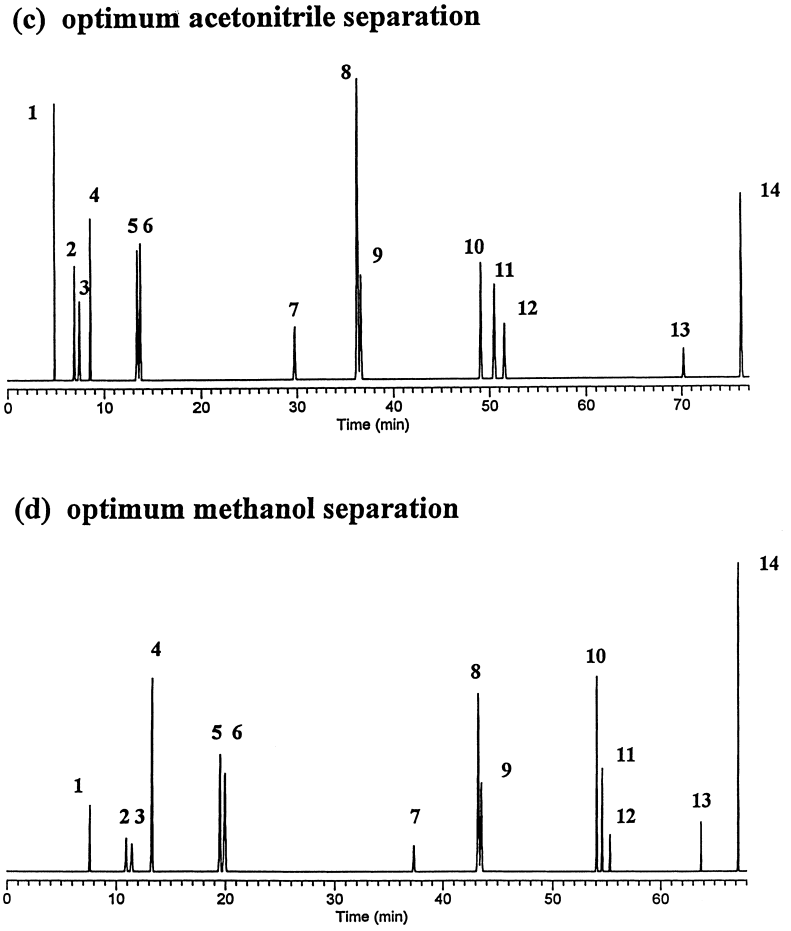


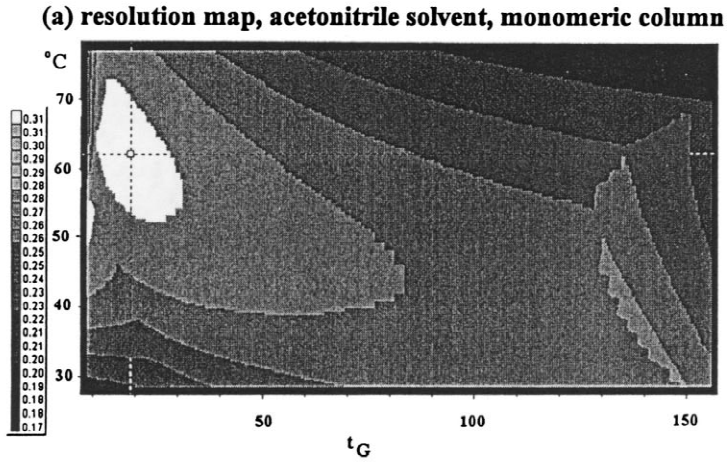
Fig. 1. (*continued*)

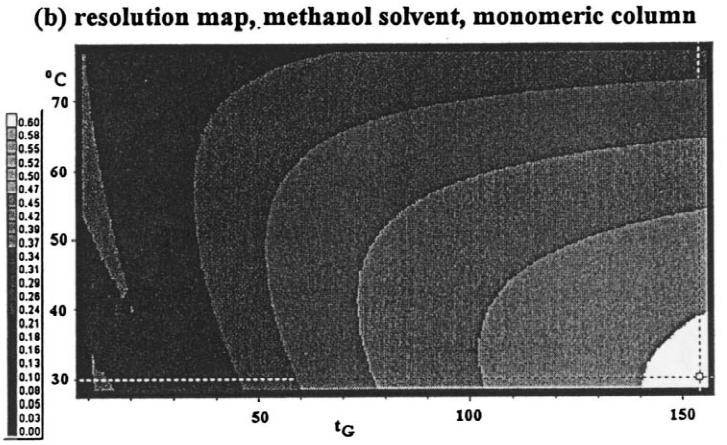
gradients in 30 and 90 min, at temperatures of 40 and 70°C. The column was a 25×0.46 cm Vydac 201TP, with a flow-rate of 1.0 ml/min. The resolution map for these latter conditions is shown in Fig. 2c, and the separation for optimum values of T (38°C) and t_G (84 min) is shown in Fig. 3c (R_s= 0.93). This resolution is still marginal, but it is superior to either of the prior two separations (Fig. 3a,b) for which T and t_G have been optimized. There are significant selectivity changes among the three runs of Fig. 3, in addition to band spacing changes for critical bands 10–13. The saturated fatty acids (Nos. 9, 14) are preferentially retained on the polymeric column (Fig. 1c), as noted in Ref. [13].

3.5.2. Algal pigments

A sub-set of the 29-component algal pigment sample described in Part I was selected for further study as a function of column type. The new sample included 12 compounds from Table 4 of Ref. [6]: Nos. 1, 2, 4–6, 12, 18, 22, 24, 25, 26 and 29. The separation of this sample using a polymeric C_{18} column gave the resolution map of Fig. 4a. Conditions for maximum resolution (48°C and 86-min gradient) gave the chromatogram of Fig. 4b (R_s= 3.55). Similar experiments were carried out with a monomeric C_{18} column; Fig. 4c shows the resolution map and Fig. 4d shows the separation for conditions (39°C, 20-min gradient) which provide maximum resolution (R_s=1.87).

A change in resolution results from this change in column, as for the previous fatty acid ester sample. However, some adjustments must be made before a valid comparison is possible. First, the ranges in





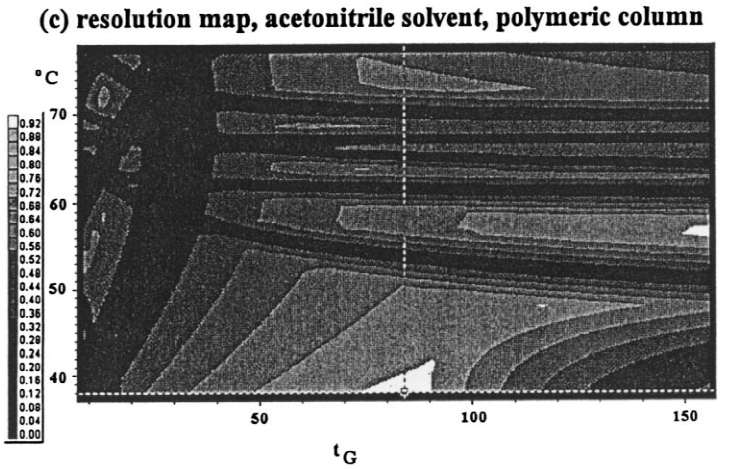


Fig. 2. Resolution maps for separation of fatty acid sample. (a) Acetonitrile solvent, monomeric C_{18} column; (b) methanol solvent, monomeric C_{18} column; (c) acetonitrile solvent, polymeric C_{18} column. Conditions: (a) 25×0.46 cm C_{18} columns, 60–100% acetonitrile in water gradients, 1.0 ml/min; (b) same as (a), except 75–100% methanol in water gradients; (c) same as (a) except for different column packing.

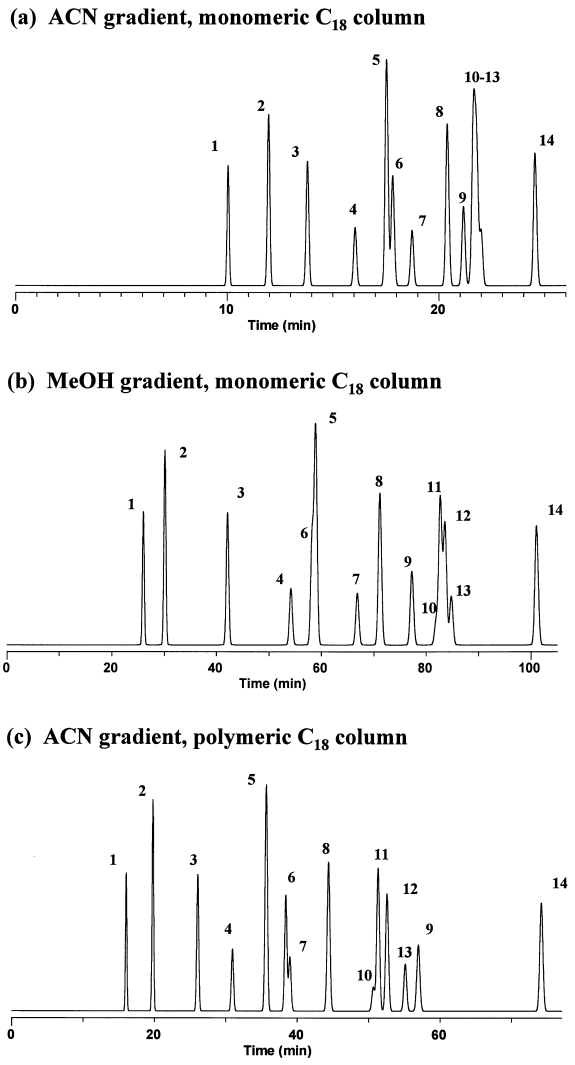


Fig. 3. Separations of the fatty acid ester sample of (1). (a) 25×0.46 cm Zorbax SB-C_{18} column; 60–100% acetonitrile in water gradient in 19 min, 62°C, 1.0 ml/min; (b) same as (a), except 75–100% methanol in water gradient in 154 min at 28°C; (c) same as (a) except 25×0.46 cm Vydac 201TP column, 60–100% acetonitrile in water gradient in 84 min at 38°C. Computer simulations based on data of Ref. [13].

temperature and gradient time differ for the two columns: $17 \leq t_G \leq 51$ min and $50 \leq T \leq 60$ for the polymeric column (Fig. 3a,b) vs. $7 \leq t_G \leq 21$ min and $40 \leq T \leq 55$°C for the monomeric column (Fig. 3c,d). The same range of temperatures should be used for comparing the columns; allowing for modest extrapolation of the original temperature range, we can select 48–57°C as a range common to the two columns. The gradient times for each column must be adjusted to provide a similar range in gradient retention k^*, which means that $t_G F/V_m$ must be comparable in each case. If the 17- to 51-min gradient times for the monomeric column are used as reference, then corresponding times for the polymeric column would be 34–102 min (for comparable k^* values). Examination of Fig. 4a and Fig. 4c shows that each of these gradient–time intervals overlaps t_G for maximum resolution, so we need not worry about differences in the range of gradient time. The resulting (comparable) values of maximum resolution are then $R_s = 3.55$ (polymeric column) and 1.73 (monomeric column, 47°C, 20-min gradient).

A second correction must be made for the differences in column length L and resulting differences in plate number: $N = 7000$ for the polymeric column and 3800 for the monomeric column. As a result, resolution for the monomeric column should be less by a factor of about $3800/7000^{1/2} = 0.74$. Taking these considerations into account, the resolution for the polymeric column ($R_s = 3.55$) must be adjusted by a factor of 0.74. The resulting adjusted resolution $R_s = 3.55 \times 0.74 = 2.62$ can now be compared with that for the monomeric column: $R_s = 1.73$. Other factors equal, resolution for the polymeric column is greater by a factor of $2.62/1.73 = 1.5$, as a result of selectivity changes between the two columns. A comparison of Fig. 4b and Fig. 4d shows significant differences in relative retention for bands 4–6 and 12, although no reversals of elution order have resulted.

Conditions for a faster separation of this 12-component sample were selected next, at the expense of baseline resolution. The predicted separation of Fig. 4e is compared with an experimental run for the same conditions in Fig. 4f. Agreement between the two chromatograms is good, except for peak 29 (β-carotene) whose predicted retention time is 3.1 min greater than the experimental value (peak 29 elutes after the gradient ends). If peak 29 is excluded, experimental vs. predicted retention times show an average deviation of 0.08 min, and retention time differences for adjacent bands (proportional to values of R_s) agree with an average deviation of ±0.06 min. This is acceptable accuracy for method development

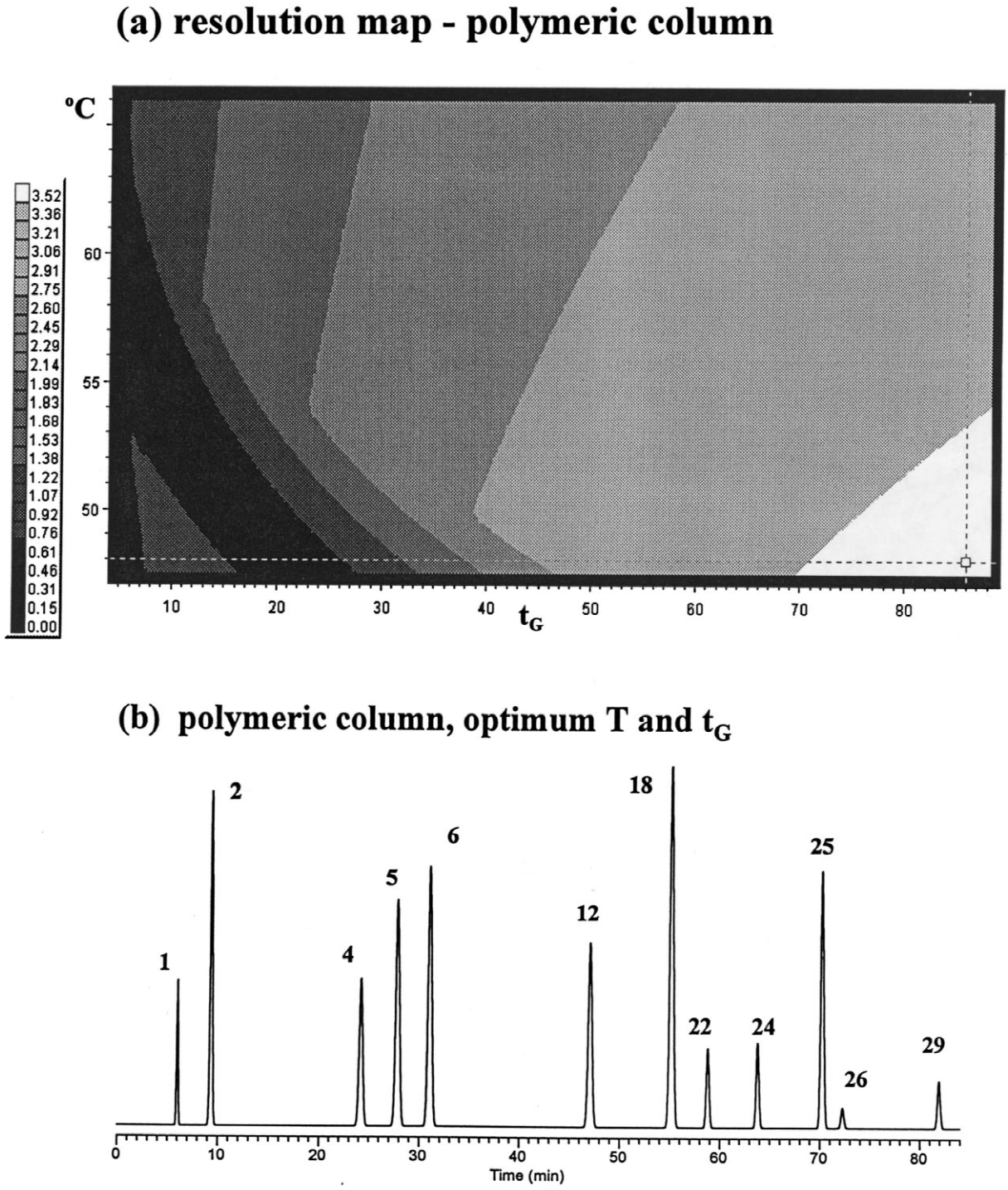
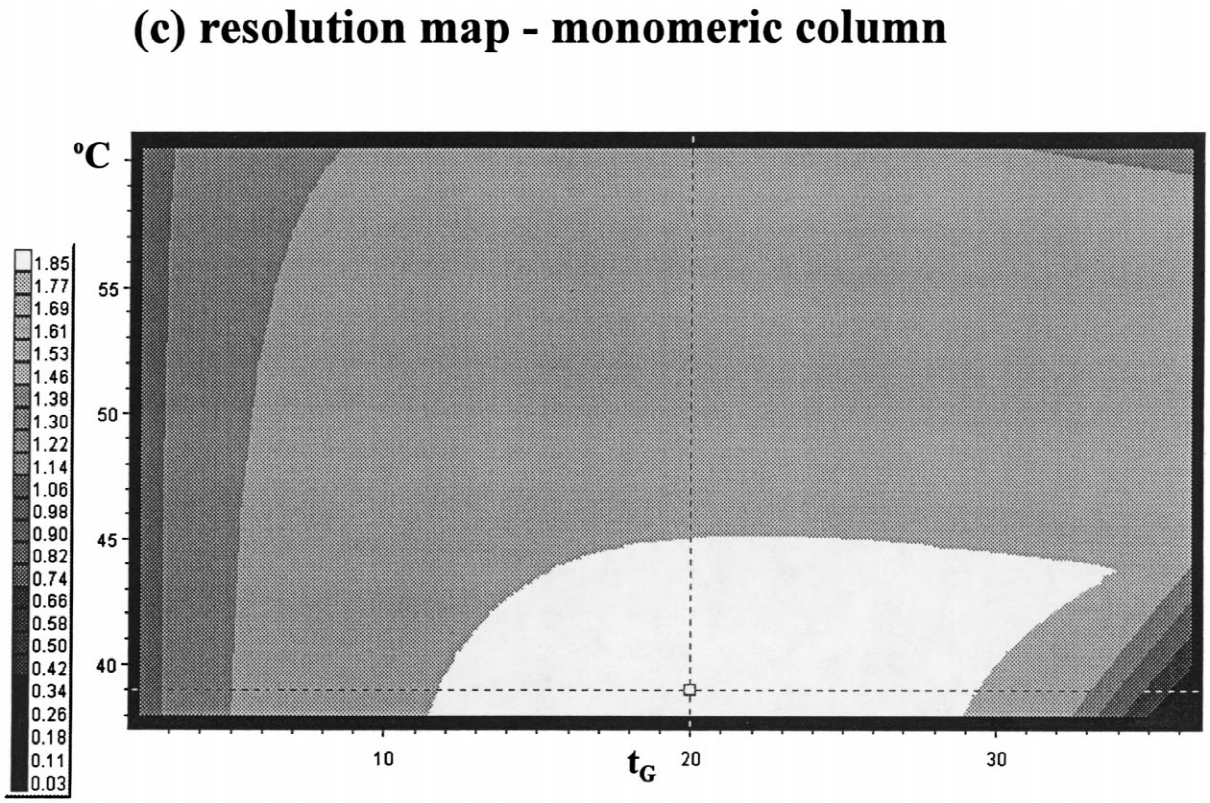


Fig. 4. Separations of 12-component algal pigment sample (a sub-set of the sample described in Table 4 of Ref. [6]). (a) Resolution map for 25×0.32 cm Vydac 201tp53 (polymeric) column; conditions: 0–100% B gradients; solvent A is 70% methanol, 28 m*M* aqueous tetrabutylammonium hydroxide adjusted with acetic acid to pH 7.1; solvent B is methanol; 0.65 ml/min; (b) optimized separation for system of (a) 48°C, 86-min gradient; (c) resolution map for 10×0.21 cm Hypersil ODS column (Shandon); conditions: same as (a) except 10–100% B gradients, 0.55 ml/min; (d) optimized separation for system of (c) 48°C, 86-min gradient; 39°C, 20-min gradient; (e) same as (d), except 39°C, 5.5-min gradient; (f) experimental run for conditions of (e). Chromatograms (b, d, e) are computer simulations.



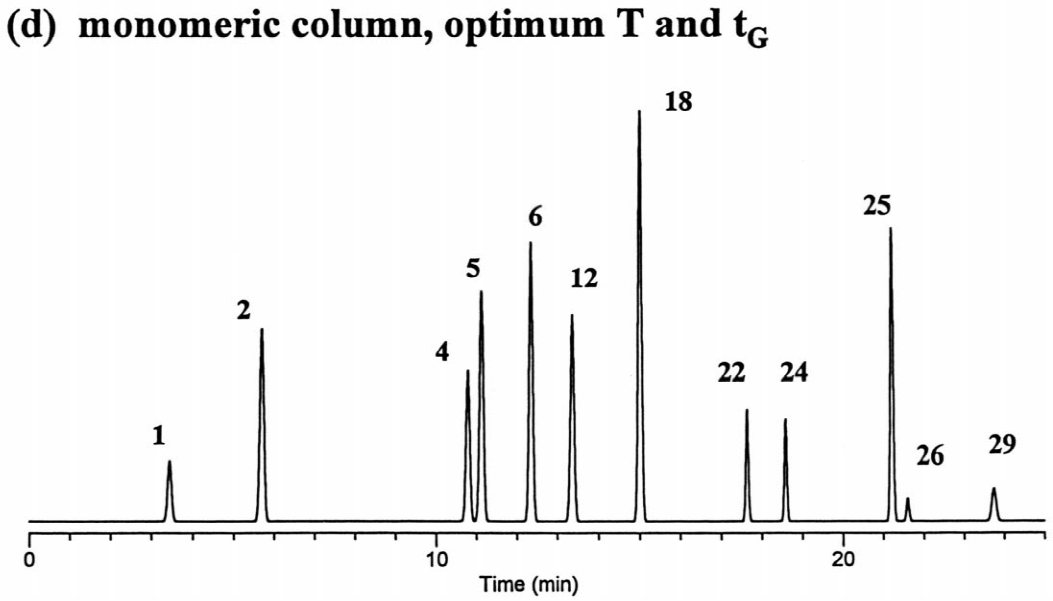


Fig. 4. (*continued*)

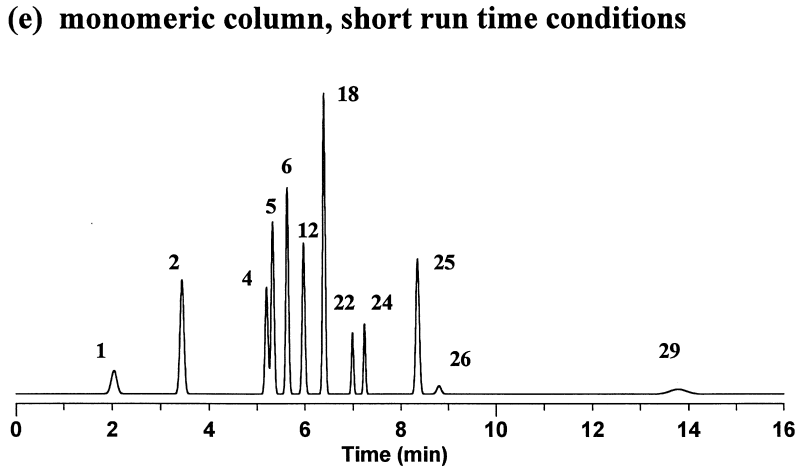


Fig. 4. (*continued*)

purposes. Note also in Fig. 4f that there is an additional peak eluting at 6.1 min.

3.6. Change of mobile phase pH

Data were reported in Ref. [14] for the separation of two different samples, where temperature, gradient time and mobile phase pH were varied. These results can be used with computer simulation to see how resolution varies with pH when T and t_G are optimized for each value of pH.

3.6.1. Substituted benzoic acids

The T and t_G optimization of this mixture of eight compounds by means of computer simulation was discussed in Ref. [15] for a mobile phase pH equal to 2.6. Fig. 5a shows the optimized separation at pH 2.6, while Fig. 5b shows the optimized separation at pH 4.3. Resolution is increased from 1.6 at pH 2.6 to

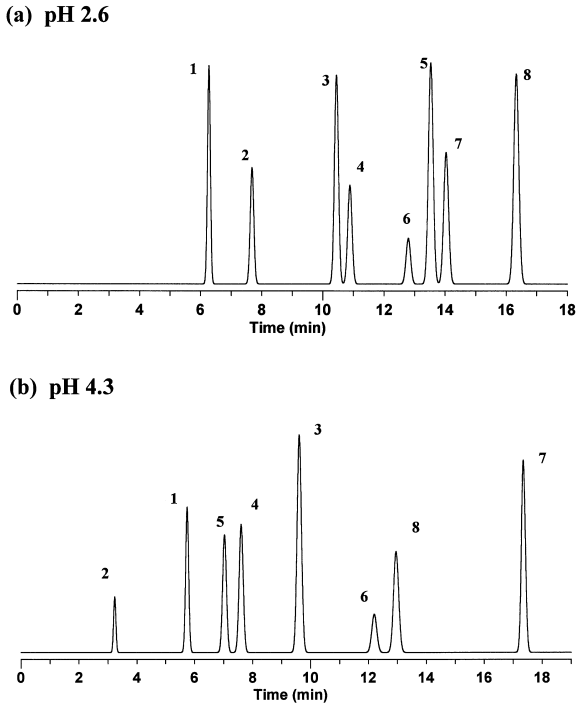


Fig. 5. Separations of 8-component substituted benzoic acid sample. (a) 15×0.46 cm, 5 μm Zorbax SB-C_{18} column; 5–50% B in 30 min; A-solvent, aqueous mixture of 25 m*M* citric acid adjusted with dibasic sodium phosphate to the desired pH value; B-solvent, acetonitrile; 77°C; 1.0 ml/min; (b) same as (a), except for mobile phase pH=4.3 and 68-min gradient at 37°C. Peak numbering as in Ref. [4]. Computer simulations based on data of Refs. [4,14].

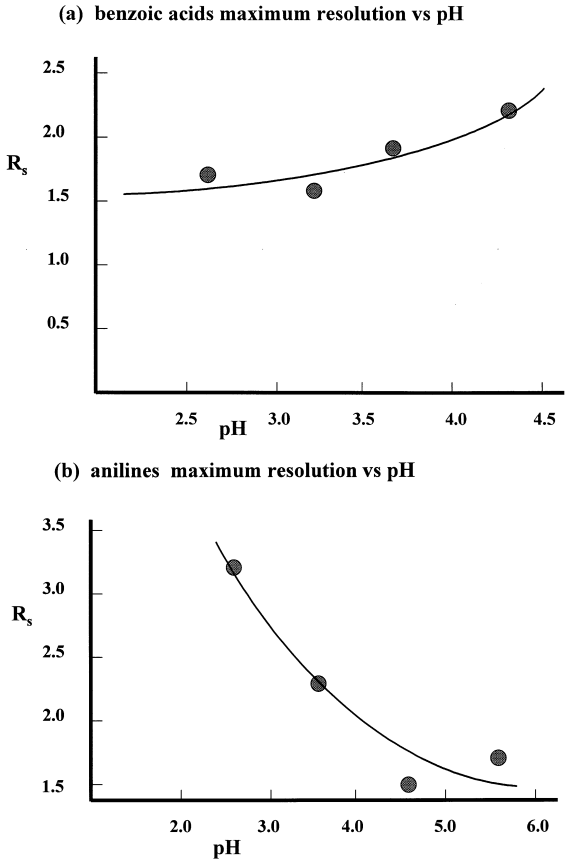


Fig. 6. Maximum sample resolution as a function of pH, assuming that temperature and gradient time are optimized for each pH. (a) Substituted benzoic acid sample (No. 11 of Table 2); (b) substituted aniline sample (No. 12 of Table 2). Computer simulations based on data of Refs. [4,14].

2.2 at pH 4.3. Again, a change in a third variable (pH in this case) has resulted in improved separation. Fig. 6a shows a plot of resolution vs. pH for optimized separations of this sample, other conditions the same.

3.6.2. Substituted anilines

The T and t_G optimization of this mixture of nine compounds by means of computer simulation was discussed in Ref. [15] for a mobile phase pH equal to 2.6. As mobile phase pH is increased, the resolution for T/t_G optimized separations decreases, as shown in Fig. 6b. In this case, the best separation is obtained for the pH of the initial separations (pH 2.6). However, depending on the pH chosen for the initial separations, higher or lower resolution could have resulted from a change in pH.

3.6.3. Testosterone derivatives

Several papers [16–19] have reported the separation of various testosterone derivatives that are included in the sample described in Table 5 of Part I [6]. In each of these previous cases, optimized gradient separations are shown based on the use of aqueous methanol–acetonitrile mobile phases. We can repeat the computer optimization of each of the samples of [16–19] using the system of Part I [6], varying temperature, gradient time and initial % B as in Table 2 for comparison with these literature

Table 3
Comparisons of optimized testosterone derivative separations by system of Ref. [6] vs. other systems

Sample No.[a]	Lit. ref.[b]	Conditions[c]		Resolution, R_s[d]	
		System of Ref. [6]	Literature	System of Ref. [6]	Literature
1,2,4–7,9, 11–13,15,17	[16]	38–54% B in 11 min; 28°C	MeOH–ACN	0.48	0.8
1,6,9,11,12, 15–17	[17]	38–54% B in 11 min; 28°C	MeOH–THF	0.58	1.2
1,6,8,9,11, 12,16,17	[18]	38–54% B in 11 min; 28°C	MeOH–ACN	0.58	0.8
1,5,6,8,9, 11–13,15–17	[19]	38–54% B in 11 min; 28°C	MeOH–ACN	0.49	1.0

[a] Sample compounds (testosterones) numbered according to Table 4 of Ref. [6].
[b] Comparative separation from the literature.
[c] Optimized conditions for system of Part I [6] vs. literature conditions.
[d] Optimized R_s for system of Part I [6] vs. literature value.

separations. These results are summarized in Table 3 for comparison with the literature separations. In every case, the use of two organic solvents is seen to result in a better separation of the sample. This is mainly the result of a better separation of compounds 11 and 12 (2α- and 2β-hydroxytestosterone) in the literature systems. The further optimization of temperature and gradient time for these separations of [16–19] should allow even better separation of the various samples of Table 3.

3.6.4. Summary of preceding examples

The effects of a change in solvent, column or pH on maximum resolution are summarized in Table 4 (assumes T and t_G are optimized for each separation condition). The change in resolution as a result of change in a third variable varies from little change (1.1-fold) to 2.9-fold, and can be either favorable or unfavorable. It is expected that when some condition is changed to a second value to improve resolution, there will be an increase in resolution for half of the separations, and a decrease for the other half. In the present examples, a change in conditions followed by re-optimizing T and t_G resulted in significantly increased resolution for two of the five samples (fatty acid esters and benzoic acids). In two other cases (algal pigments, anilines), resolution decreased. The data of Table 4 thus confirm the potential value of changing a third variable and re-optimizing T and t_G, when an initial optimization of T and t_G falls short of acceptable resolution. An unanswered question is whether a full optimization involving all combinations of T, t_G and the third variable will generally result in a significant further increase in maximum resolution. The data of Fig. 6 suggest not, in that resolution is seen to vary regularly (with minor scatter of data points about a smooth curve) between extreme values of the third variable (pH in these examples). In any case, a full optimization of pH, T and t_G would require a very large number of experimental runs and is impractical (and unnecessary) for most samples.

3.7. Change in a third condition

The choice of a change in the third variable is critical to the success of the present strategy and is discussed below.

3.7.1. Change of pH

For samples composed of acids and/or bases, a change in pH or the use of ion-pairing is a good first step. The benzoic acid and aniline samples of Table 4 show an average change in optimized resolution of 1.7-fold, and this is probably typical. However, it is important to choose a change in pH which is likely to result in large changes in sample ionization and resulting maximum changes in band spacing. Compounds in the benzoic acid and aniline samples have pK_a values of 2.0–3.8 [20], so large changes in

Table 4
Change in maximum resolution as a result of a change in a third variable for separations that have been optimized for temperature and gradient time (both initially and finally)

Sample	Variable changed[a]	Effect on optimized resolution		
		R_s[a]		Change in R_s[b]
		1	2	
Acrylate monomers	Acetonitrile (1)			
	Methanol (2)	1.38	1.31	1.1-fold
Fatty acid methyl	Acetonitrile (1)			
esters	Methanol (2)	0.32	0.61	1.9-fold
	Polymeric column (1)			
	Monomeric column (2)	0.93	0.32	2.9-fold
Algal pigments	Polymeric column (1)			
	Monomeric column (2)	2.6	1.7	1.5-fold
Substituted benzoic	pH 2.6 (1)			
acids	pH 4.3 (2)	1.6	2.2	1.4-fold
Substituted anilines	pH 2.6 (1)			
	pH 4.6 (2)	3.2	1.6	2.0-fold
		Average		1.8-fold

[a] ''1'' and ''2'' refer to changed variables in first and second series of experiments (four runs with T and t_G varying).
[b] No weight is given to which variable (1 or 2) was tried first; therefore the change in R_s is always ≥ 1.0.

sample ionization are possible for changes in pH in the range $2<pH<5$, and this is reflected in the plots of Fig. 6. *For ionizable samples, it is recommended that initial experiments be carried out at low pH (e.g., pH=2–2.5* [1]*), in order to minimize silanol effects and avoid resulting peak distortion and broadening*. At low pH, most bases will be ionized and most acids will be non-ionized (although not the case for the anilines sample of Fig. 6b). However, it should be noted that many silica-based reversed-phase columns are unstable at low pH and higher temperatures (see Ref. [1], pp. 193–203). Unless so-called ''sterically protected'' columns are used, it is advisable to use only longer-chain-length packings (e.g., C_{18}), maintain pH>2.5, and avoid temperatures >50°C.

If a change in selectivity is to result from a change in pH, the change in pH must be large enough to change sample ionization significantly. This usually means a pH increase of at least two units, starting from pH≈2–2.5; if the sample pK_a is >>4, then a larger increase in pH will be necessary in order to change selectivity appreciably.

3.7.2. Change of solvent type

Because of the instability of tetrahydrofuran (THF, air oxidation) and its higher UV cut-off wavelength, the use of this solvent is often avoided. On the other hand, it appears that a change from either methanol or acetonitrile to THF is more effective in changing selectivity than is a change from methanol to acetonitrile [3]. Thus, whereas both methanol and acetonitrile as solvents gave similar separations of bands 8/9 of the acrylate monomer sample, it is possible that the use of THF would have resulted in better resolution.

3.7.3. Change of column type

As noted in Ref. [3], a cyano column is maximally different from a C_8 or C_{18} column, making it a first choice for a change of column type if initial experiments have used an alkyl-silica column. This selectivity difference of the cyano column can be enhanced by using a wide-pore version, which further reduces sample retention (and increases changes in α) vs. that for a C_8 or C_{18} column [10]. There are exceptions to this recommendation, however. For

samples differing in molecular shape, a polymeric alkyl-silica column is more likely to create useful changes in selectivity, as illustrated by the fatty acid ester and algal pigments samples of Table 4. Other examples of such samples include polycyclic aromatic hydrocarbons [11] and carotenoids [12]; the algal pigments sample contains several carotenoids, among other compounds. For samples differing in molecular shape, it is usually best to begin method development with a polymeric (as opposed to monomeric) alkyl-silica column.

3.8. Best separation of the 14 samples of Table 2

The initial optimization of T and t_G for these 14 samples resulted in the separation of nine samples with $R_s>1$. Based on the present results and discussion, further improvements in resolution are possible for the remaining five samples (Nos. 4, 5, 7, 10, 13).

3.8.1. Algal pigments sample (No. 4)

No attempt was made to improve the separation of this 29-component sample ($R_s=0.7$), other than to optimize initial % B (which gave no increase in resolution, Table 2). There was no difficulty in separating the 12-compound sub-set of this sample (Fig. 4).

3.8.2. Fatty acid methyl esters (No. 5)

The use of a polymeric C_{18} column in place of a monomeric column resulted in an increase in resolution for this sample from $R_s=0.3$ to 0.9. The optimization of initial % B, T and t_G for this sample and column then provides $R_s=1.1$ (52–90% B, 39°C, 143-min gradient). This separation is shown in Fig. 7a.

3.8.3. Testosterones sample (No. 7)

A change in solvent (either methanol or THF) with re-optimization of T and t_G is expected to provide some increase in resolution. It is also encouraging that simultaneous optimization of initial % B, T and t_G (Table 2) doubled the resolution of this sample, compared to the case where initial % B was not varied. However, this sample is a good example of the limited ability of T/t_G optimization to provide adequate control over band spacing for a mixture of isomers. Possibly the use of an isomer-specific column packing such as cyclodextrin-silica, combined with variation in T and t_G, would lead to acceptable separation, but even this seems doubtful.

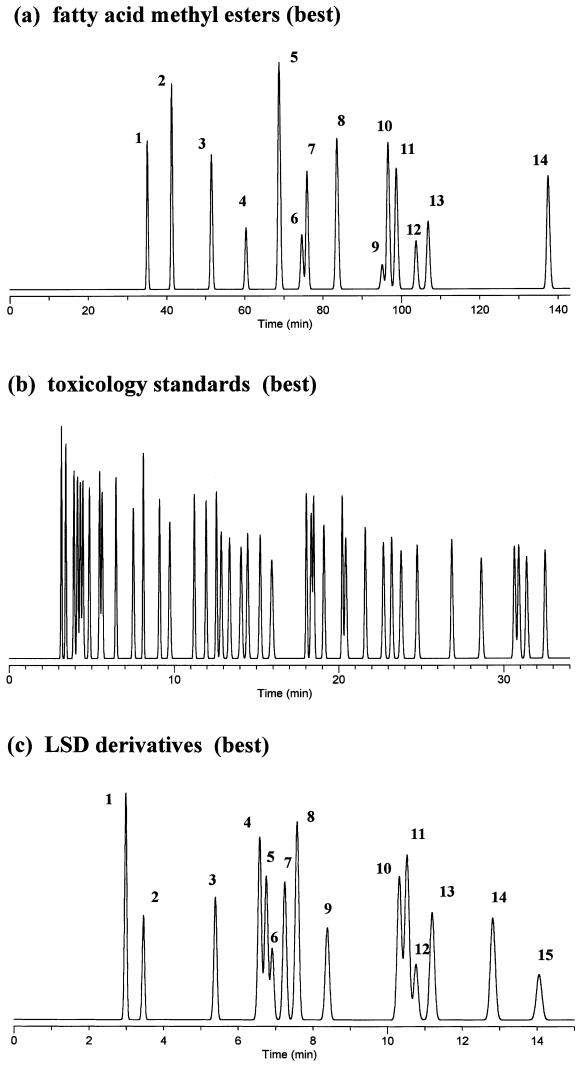


Fig. 7. Final separations of three samples. (a) Fatty acid esters sample; same as in Fig. 3c, except 52–90% B in 143 min, 39°C; (b) toxicology standards sample; same as in Fig. 11b of Part I [6], except 12–79% B in 33 min, 30°C; (c) LSD sample; same as in Fig. 12c of Part I [6], except for 20–88% B in 56 min, 56°C.

3.8.4. Toxicology standards sample (No. 10)

The only improvement attempted for this separation was the simultaneous optimization of initial % B, T and t_G, which resulted in an increase in

resolution from R_s=0.7 to 0.9. The latter separation is shown in Fig. 7b.

3.8.5. LSD derivatives (No. 13)

The only improvement attempted for this separation was the simultaneous optimization of initial % B, T and t_G, which resulted in a modest increase in resolution from R_s=0.8 to 0.9. The latter separation is shown in Fig. 7c.

To summarize, of the original 14 samples, it has been possible to separate 12 of these samples with $R_s \geq 0.9$ and one sample (algal pigments) with R_s= 0.7. A modest change in column conditions could reasonably be expected to give $R_s > 1$ for these 13 samples. The only unsuccessful separation was that of the testosterone isomers, and the data of Table 3 suggests that the use of another solvent might have improved this separation.

3.9. Suggested method development approach

It is convenient to carry out method development in a gradient mode, since computer simulation allows predictions for corresponding isocratic separations at any time. One advantage of selecting temperature and gradient time as variables to control selectivity is that only four initial experiments are needed for subsequent optimization of these and other conditions by means of computer simulation. We believe that many samples can be separated satisfactorily, on the basis of these four initial experiments alone. When this proves not to be the case, method development can proceed by changing a third variable and re-optimizing T and t_G (making a total of eight runs). The evidence summarized here and in Part I [6] suggests that most samples will not require further changes in conditions.

A preceding paper [21] has suggested an alternative method development procedure which relies on solvent type and strength selectivity, rather than changes in T and t_G. This approach, like the use of T and t_G as variables, is convenient (six runs to start method development), amenable to the use of computer simulation, and does not create problems in the final method. At this time it is not clear when or if the procedure of Ref. [21] will be preferred. This is a question we are pursuing.

4. Conclusions

The advantage of temperature (T) and gradient time (t_G) as variables to control selectivity and optimize separation can be enhanced in various ways. Changes in column conditions or initial % B, followed by re-optimizing T and t_G, can provide modest increases in resolution – by a factor of up to 1.5-fold. The effects of changes of this kind can be predicted by computer simulation and do not require additional experiments. A change in some other variable (solvent or column type, pH) followed by re-optimization of T and t_G can be more effective, but requires four additional experiments for each change of variable. The use of temperature and gradient steepness for controlling band spacing and resolution might be assumed to be less effective than other variables such as solvent or column type, because these latter variables are individually better able to alter separation selectivity [3]. However, this may not be true for simultaneous changes in T and t_G as used in the present and preceding paper [1]. This question is the subject of ongoing study.

Using the present approach to HPLC method development, it was possible to separate 13 of 15 ''difficult'' samples with $R_s \geq 0.9$. It is reasonable to conclude that most other samples can be separated with $R_s \gg 1$ by the same procedure.

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Appendix A

Criteria for the applicability of segmented-gradients for improving resolution

If the values of φ at elution for two critical band-pairs are $\varphi_{i/j}$ and $\varphi_{p/q}$, and if

$$\varphi_{p/q} - \varphi_{i/j} > (1 + 0.9b)/S \quad (1)$$

then a maximum increase in resolution can be expected when using a segmented gradient for this purpose (for the derivation of Eq. (1), see Ref. [7] and note that a term $0.9t_0$ should be added to the right-hand-side of Eq. 6 of Ref. [7]). For small-molecule samples, with molecular masses <500, an average value of $S \approx 4$ can be assumed [22] so that

$$\varphi_{p/q} - \varphi_{i/j} > (1 + 0.9b)/4 \quad (2)$$

For reasonable retention of the sample ($0.5 < k^* < 20$, $1.7 > b > 0.04$) this leads to the approximate relationship

$$\varphi_{p/q} - \varphi_{i/j} > 0.5 \quad (3)$$

if a segmented gradient is to have its maximum effect in increasing sample resolution. That is, the two critical band-pairs should elute at mobile phase compositions that differ by 50% B.

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