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Reversed-phase liquid chromatographic separation of complex samples by optimizing temperature and gradient time II. Two-run assay procedures

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Abstract

By optimizing column temperature T and gradient time t_G, complex samples can often be separated by means of reversed-phase high-performance liquid chromatography (RP-LC). Conclusions reached in Part I suggest that the complete separation of such samples will be difficult, however, when more than 15–20 components are present in the sample. An alternative approach is to carry out two separations with different conditions (T, t_G) in each run. The combination of results from these two runs then allows the total analysis of the sample, providing that every sample component is adequately resolved in one run or the other. Examples of this approach, carried out by means of computer simulation, are shown here for several samples of varying complexity. Also considered is the ability of a single separation where T and t_G are optimized to enable the separation and analysis of one or more individual sample components from complex mixtures (e.g., drugs in animal plasma), including the resolution of isomeric compounds from each other.

Keywords: Optimization; Temperature effects; Gradient elution; Computer simulation; Resolution

1. Introduction

A common goal is the separation of all analytes in a sample with some minimum resolution R_s for the ''critical'' or least resolved band pair. The probability of an adequate separation decreases rapidly, as the number n of analytes increases, and in [1] it was shown that the optimization of temperature T and gradient time t_G is unlikely to be successful when n is greater than 15–20. In the past, the total analysis of complex samples by liquid chromatography has involved various expedients. One approach is an initial separation of the sample into several fractions, each of which is simple enough to be resolved by a second separation that offers different selectivity [2,3]. A similar procedure is the use of column-switching to achieve the same result without manual intervention [4–6]. Another option combines a single high-performance liquid chromatographic (HPLC) separation with selective detection [7,8]; e.g., using diode-array or mass spectrometry detection. A possible future means of separating complex samples is

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the use of capillary electrochromatography (CEC), which may eventually allow a major increase in column efficiency (N) and peak capacity. Finally, two or more separations of the total sample with differing HPLC conditions may result in the separation of every sample component in one or the other of these runs. A total analysis of the sample can then be achieved by combining results from the two separations [9]. The present study was directed mainly toward a better understanding of the latter approach, where column temperature T and gradient time t_G differ between the two runs.

Part I [1] demonstrated the effectiveness of T and t_G as separation variables for the complete separation of samples where n does not exceed a certain value ($n=15–20$ for most samples). That study also suggests that these two variables (T, t_G) are similarly effective as for the use of solvent optimization for resolving complex samples. However, a more common goal in the analysis of complex samples is *not* the total separation of the sample, but rather the adequate resolution of one or more compounds of interest. A question arises in this connection: how effective is the optimization of T and t_G for the separation of *individual* compounds in complex mixtures? As will be seen, this issue is also related to the potential applicability of 2-run procedures for the *total* separation of complex samples. The present study addresses both objectives.

2. Experimental

All materials, samples and experimental procedures are described in [1]. Computer-simulation software (DryLab *for Windows*, version 2.0; LC Resources) was used for the various ''experiments'' described in the present paper. Fig. 1 illustrates the use of computer simulation to create resolution maps (a,b,d,f) and simulate separations (c,e,g) for some or all components of a 48-component sample. Resolution R_s is defined for maps as in Fig. 1 to mean the resolution of a critical pair in the sample, where at least one of the latter two compounds is of interest (i.e., is an analyte). When the separation of all sample components is of interest, the resolution map describes R_s as a function of T and t_G for the poorest-resolved band-pair in the sample (e.g. Fig. 1a). *Partial resolution maps* (e.g. Figs. 1b,d,f) describe the resolution of bands of interest from each other and the remainder of the sample, while ignoring the separation of other bands from each other.

3. Results and discussion

3.1. Separating a single analyte from a complex mixture

Fig. 2 shows the simulated separation of a 48-component sample (mixture of toxicology and internal standards; sample 24 of Table 2 of [1]) after T (47°C) and t_G (64 min) have been optimized. This separation can be compared to the simulation of Fig. 1 of [1], where the same sample was separated using arbitrary (non-optimized) conditions: T=50°C and $t_G=40$ min. In the latter separation, 13 compounds were poorly separated ($R_s<1$), with complete overlap ($R_s=0$) for one band-pair. In the optimized separation of Fig. 2, only nine compounds are poorly resolved ($R_s<1$), and all peaks have $R_s>0.4$. Thus, optimized values of T and t_G result in an improved separation of this sample (Fig. 2), but this result is still unsatisfactory ($R_s \ll 1$). Later, we will compare this result with a corresponding 2-run assay, which can provide $R_s \geq 0.8$ for all peaks.

3.1.1. Resolution maps for selected peaks

A resolution map for the separation of Fig. 2 is shown in Fig. 1a, where the cursor (cross-hair) marks conditions for maximum sample resolution ($R_s=0.4$). The latter map describes the resolution of the poorest-resolved (''critical'') band pair as a function of T and t_G. We can also select resolution maps of other kinds. For example, consider band-pair 27/28 in Fig. 2, where $R_s=0.5$. Because the conditions of Fig. 2 have been selected for maximum sample resolution as defined by Fig. 1a, it might appear that $R_s=0.5$ represents the best possible separation of bands 27 and 28 in this system (for any value of T or t_G) — but this is not the case. If we select *only* bands 27 and 28 for maximum resolution (separation from all other compounds and from each other), the resolution map of Fig. 1b results. For optimized conditions of $T=70$°C and $t_G=28$ min, a maximum resolution $R_s=2.1$ for bands 27 and 28 is predicted

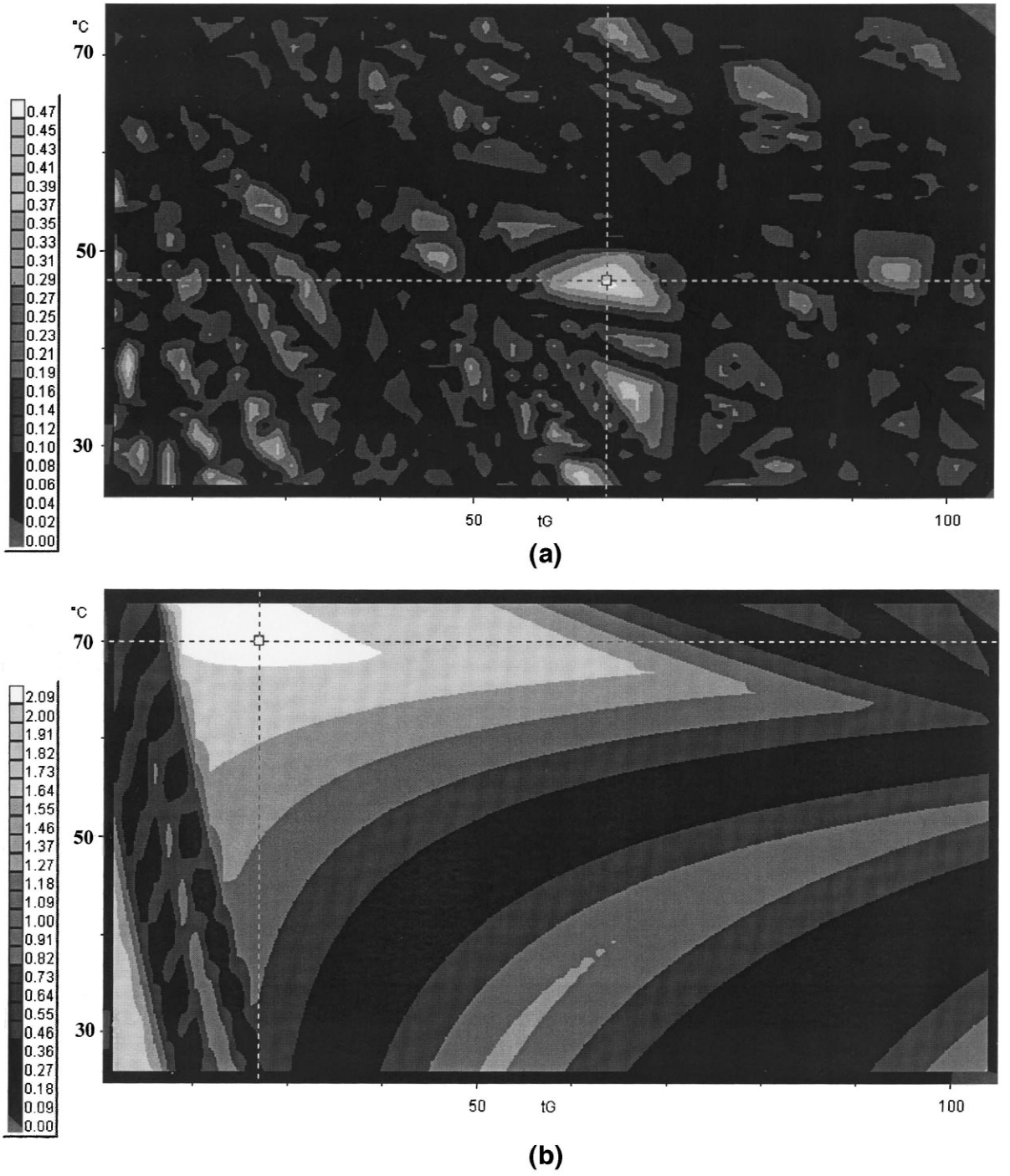


Fig. 1. Critical resolution maps for 48-component sample of Fig. 2. (a) Map for all components of the sample; (b) map for compounds Nos. 27 and 28 of Fig. 2; (c) best separation of bands 27 and 28 after optimizing T=70°C and t_G=27 min; (d) map for compound No. 28 of Fig. 2; (e) best separation of band No. 28 after optimizing T=74°C and t_G=38 min; (f) map for compounds Nos. 27 and 28 alone (no other compounds present in the sample); (g) best separation of compounds Nos. 27 and 28 (alone) after optimizing T=28°C and t_G=103 min. Cursor in maps marks conditions for maximum R_s. See text for details. t_G axes are in min. (*Continued on next page*)

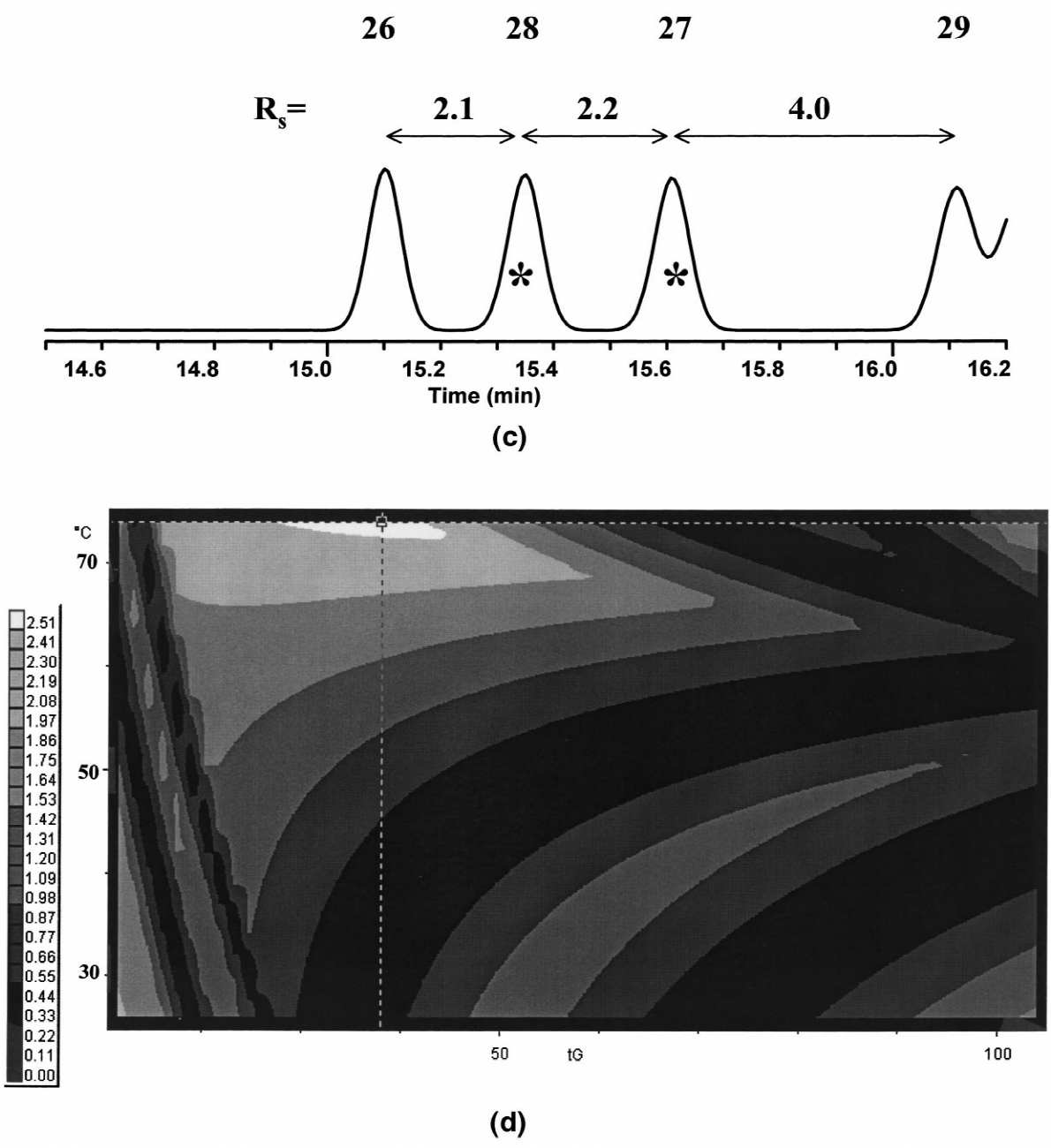
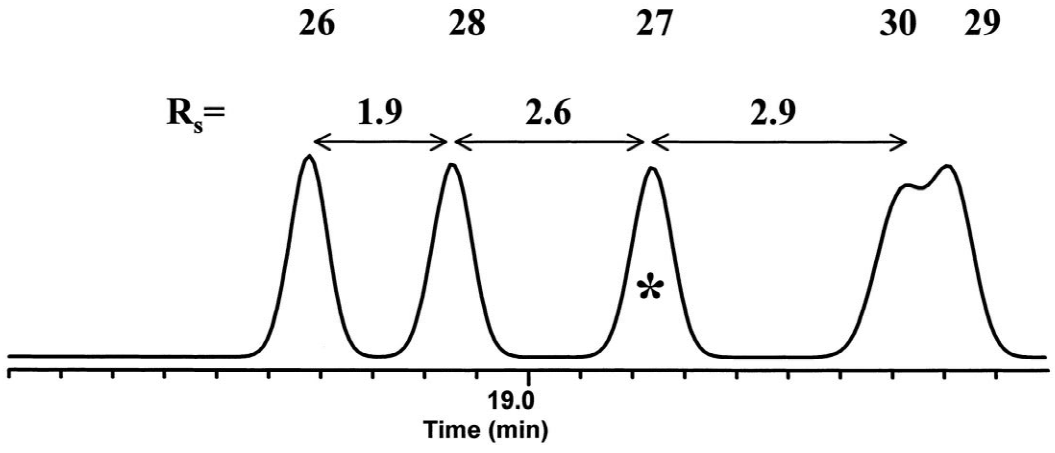


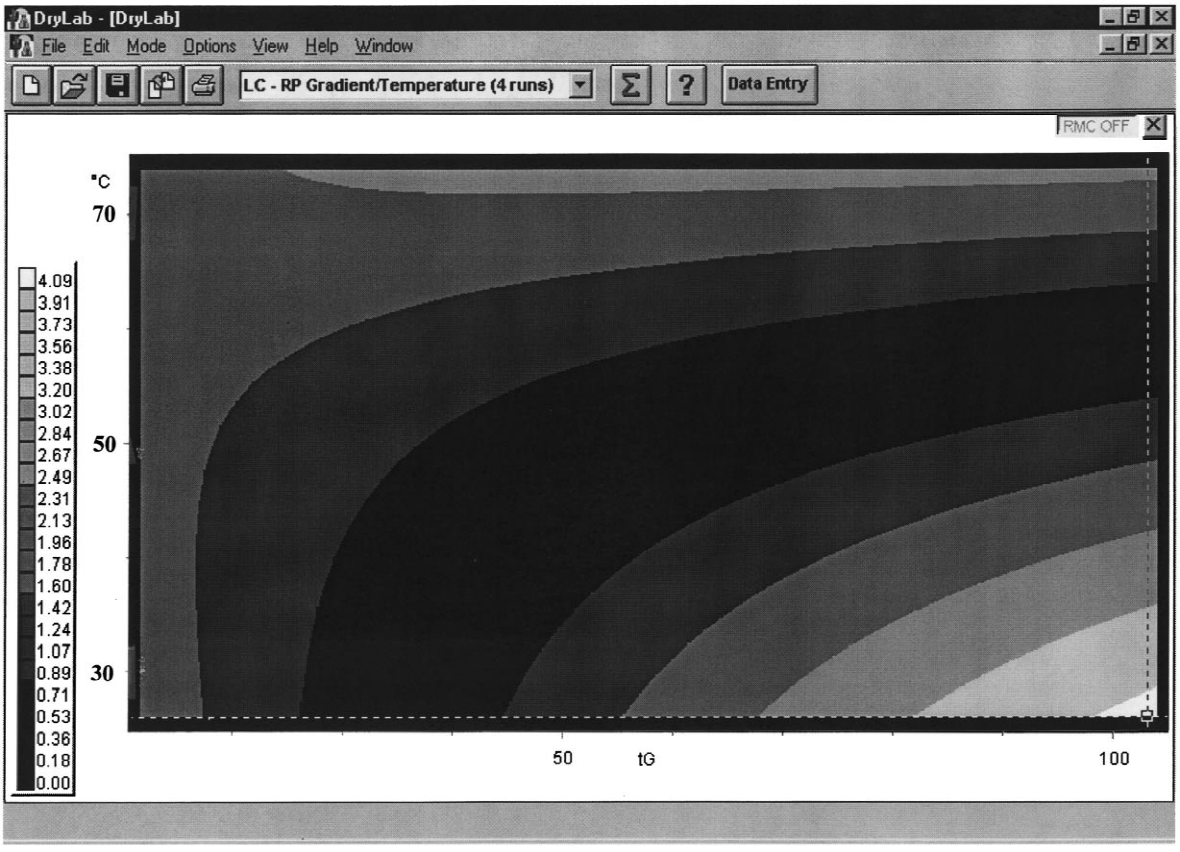
Fig. 1. (*continued*)

(see Fig. 1c), although the separation of some other bands will be much poorer for these conditions. Note also the reversal of bands 27 and 28 in the chromatogram of Fig. 1c, vs. that of Fig. 2.

Still another resolution map results, if we wish to optimize the separation of a *single* compound in the sample. This is illustrated in Fig. 1d, for the maximum resolution of peak 27. For optimized conditions



(e)



(f)

Fig. 1. (*continued*)

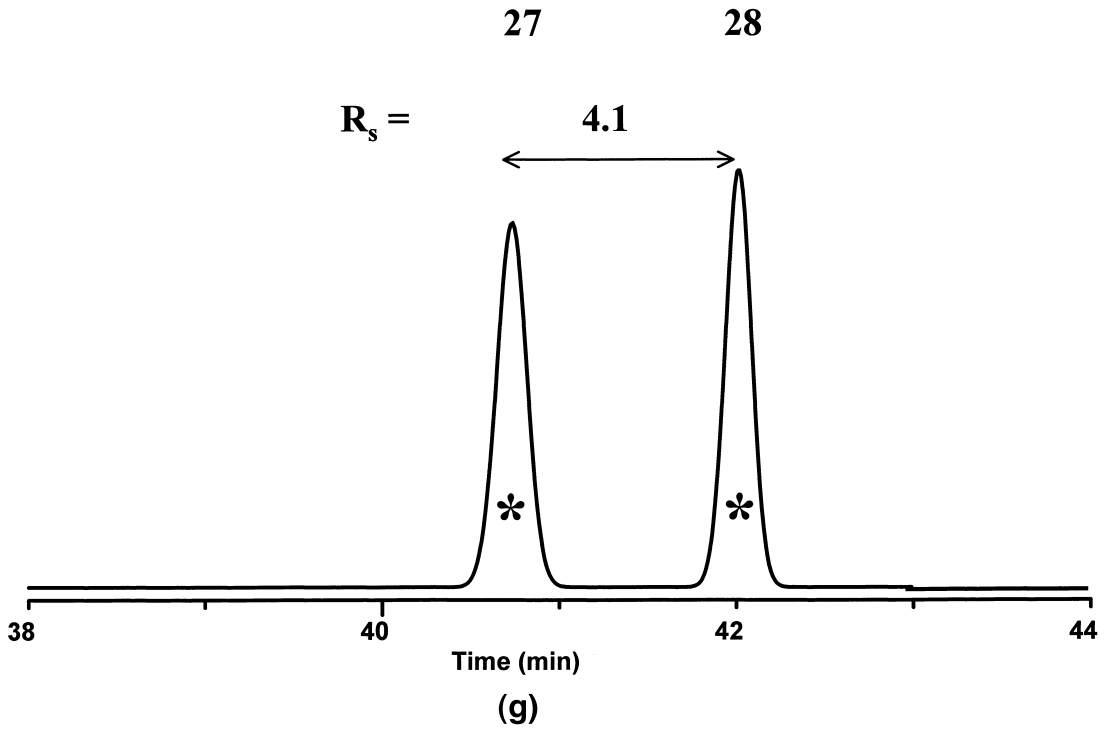


Fig. 1. (*continued*)

of T=74°C and t_G=38 min, the resolution of this compound from adjacent peaks 28 and 30 is increased further, to R_s=2.6 (Fig. 1e).

A final resolution map for bands 27/28 results if we are concerned only with their separation from each other (assumes only these two bands present in the sample). This resolution map is shown in Fig. 1f, with the optimum separation (R_s=4.1) shown in Fig. 1g. The various kinds of resolution maps as in Fig. 1 can be useful for different situations discussed below.

3.1.2. Separating individual compounds: "resolution lists"

Single-peak resolution maps as in Fig. 1d can be used to obtain the maximum possible resolution for each sample component. It can be useful to list such resolution values for a given sample in increasing order, as in Table 1 for the 48-component sample of Fig. 2. Interestingly, while the *best* separation of the total sample is just R_s=0.4, the *worst* resolution of any single compound in Table 1 is R_s=1.7, when conditions are optimized for the separation of each (single) compound. Furthermore, out of a total of 48 compounds, all but three compounds can be separated with $R_s \geq 2.5$. Thus, the baseline separation of any of these 48 compounds is possible, and because of the large potential resolution of any given band, all but a few of these compounds could be readily isolated in substantial quantities by preparative liquid chromatography; see the discussion of [10]. Table 1 also informs us that the maximum possible resolution of the total sample (e.g. by carrying out two or more runs with different values of T and t_G) is R_s=1.7.

Table 2 summarizes results as in Table 1 for 24 different samples described in Table 2 of [1]. For each sample (and in some cases variation of a third variable other than T and t_G), Table 2 shows how many compounds could not be separated with a resolution of at least R_s=0.5, 1.0 or 1.5, respectively, after optimizing T and t_G for that compound (as in Fig. 1d,e). We might expect that the proportion of poorly resolvable compounds in the sample will increase as n increases, and this is roughly true. A

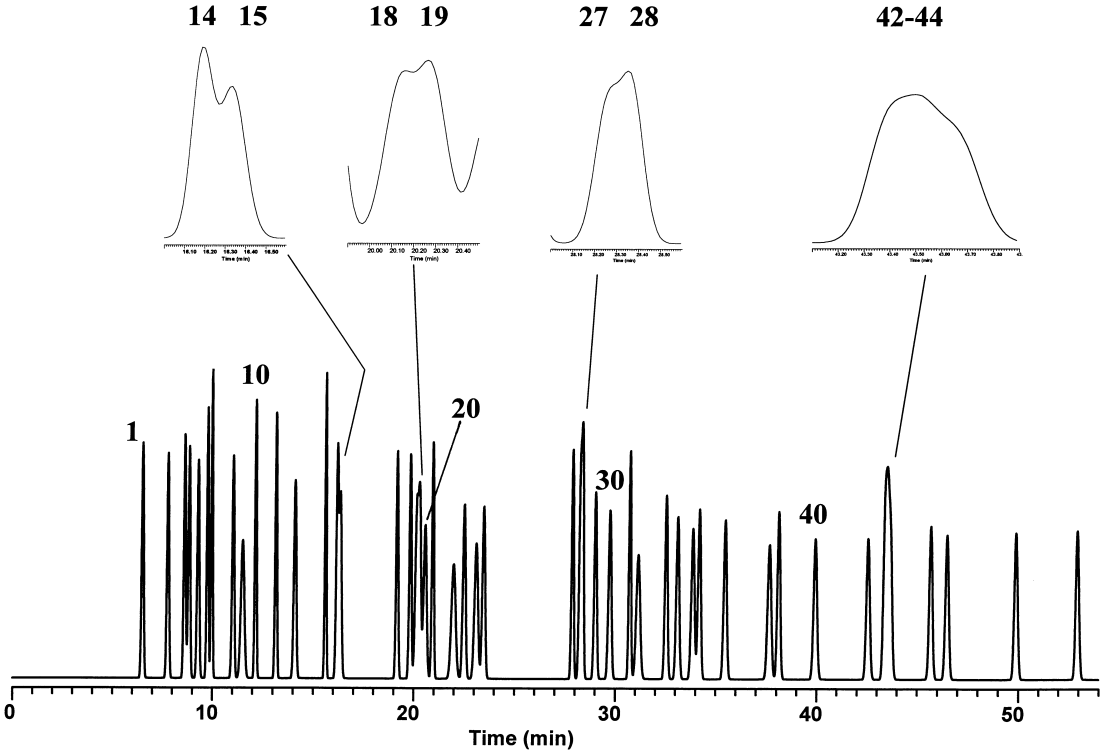


Fig. 2. Separation of 48-component sample (toxicology and internal standards) with T (47°C) and t_G (64 min) optimized. Conditions predicted by resolution map of Fig. 1a.

Table 1
Optimized separation of individual compounds from sample of Fig. 2 (40 toxicology standards plus eight internal standards; sample No. 24 of Table 2). Only the poorest-resolved 13 peaks are listed

Peak no. (Fig. 1)	Compound	R_s	t_G (min)	T (°C)
42	Phenylbutazone	1.7	103	74
43	Mefenamic acid	1.7	103	74
28	Imipramine	2.1	24	74
22	1-Nitrobutane	2.5	53	71
24	Acetophenone	2.5	29	26
27	Cortisone	2.5	37	74
26	Desipramine	2.6	36	40
20	Salicylic acid	2.8	25	50
9	1-Nitropropane	2.8	32	53
44	Biphenyl	2.9	36	26
19	Apobarbital	2.9	23	27
7	*N*-Acetylprocainamide	3	89	68
18	3-Indolecarboxaldehyde	3	25	26

marked exception to this generalization results, however, for the most complex sample (No. 24 with $n=48$), where individual compounds can each be separated with $R_s \geq 1.7$ (Table 1). A more important cause of poorly resolved *individual* peaks (other than a larger value of n) is the presence of compounds in the same sample of similar molecular structure; e.g., isomers. Samples 5, 9, 10 and 14 in Table 2, each of which contain one or more isomer pairs, are noteworthy for their larger proportion of poorly resolved compounds. The separation of isomers and other structurally-similar molecules from each other (by RPLC with optimization of T and t_G) is examined further in a later section.

Table 2 and other considerations strongly suggest that optimizing T and t_G is a highly promising approach for the separation of one or a few compounds from structurally unrelated compounds in complex mixtures. Well-known examples of such separation problems include the assay of (a) drugs

Table 2
Ability of optimized temperature T and gradient time t_G to separate individual components of different samples. For a further description of these samples and separation conditions, see Table 2 of [1]. See text for further details

Sample[a]		Separation conditions[a]	Ref.	R_s[b]	n[c]	No. of peaks with indicated resolution R_s[d]		
						$R_s<0.5$	$R_s<1.0$	$R_s<1.5$
1	Substituted benzoic acids	pH 2.6	[11,16]	1.7	8	0	0	0
		pH 3.2		1.6				
		pH 3.7		2.17	6	0	0	0
		pH 4.3		2.16	6	0	0	0
2	Substituted anilines	pH 2.6	[11,16]	6.36	5	0	0	0
2		pH 3.6		2.32	9	0	0	0
2		pH 4.6		1.55	9	0	0	0
2		pH 5.6		1.72	9	0	0	0
3	Herbicide impurities		[11]	4.91	9	0	0	0
4	Pharmaceuticals		[11]	5.91	9	0	0	0
5	Corticosteroids		[11]	1.07	9	0	0	4
6	Synthetic organics		[11]	2.58	11	0	0	0
7	algal pigments	col-A	[16]	3.27	12	0	0	0
7		col-B		1.32	12	0	0	0
8	Herbicides		[11]	1.57	13	0	0	0
9	LSD derivatives		[11]	0.77	13	0	2	4
10	Fatty acid esters[e]	ACN, col-B	[11]	0.32	13	2	2	4
10		MeOH, col-B	[16]	0.61	14	0	6	6
10		ACN, col-A	[16]	0.93	14	0	0	2
11	Acrylate monomers[e]	ACN	[11]	1.38	14	0	0	2
11		MeOH	[16]	1.31	14	0	0	2
12	Benzoic acids+anilines	pH 2.6	[17]	1.29	14	0	0	0
13	Basic drugs		[15]	1.95	15	0	0	0
14	Testosterones[e]		[11]	0.20	17	3	7	8
15	Herbicides	pH 2.7	[1]	0.64	19	0	2	2
		pH 3.5	[1]	0.69	19	0	0	2
16	Recombinant human growth hormone digest		[18]	2.97	20	0	0	0
17	Synthetic organics	column 1, ACN	[1]	0.54	21	0	4	6
		column 2, ACN	[1]	0.73	21	0	2	2
		column 1, MeOH	[1]	1.09	22	0	0	4
18	Nonbasic drugs		[15]	1.14	25	0	0	0
19	Algal pigments		[11]	0.71	29	0	0	0
20	Synthetic organics		[1]	0.53	33	0	0	4
21	rtPA digest		[19]	0.48	37	0	0	3
22	Basic+nonbasic drugs		[15]	0.65	40	0	0	0
23	Drug sample	ACN	[1]	0.61	47	0	0	2
		MeOH	[1]	0.43	47	0	2	5
24	No. 22+nitroalkane standards		[15]	0.43	48	0	0	0

[a] Same samples of Table 2 of [1]; separation conditions for maximum resolution of total sample (column, mobile phase organic solvent) are noted when these (and only these) have been changed for a particular sample.
[b] Maximum total sample resolution (single run) for optimized temperature and gradient time.
[c] Number of sample components.
[d] Number of sample compounds that cannot be separated individually with the indicated resolution, when T and t_G are optimized.
[e] Samples which are difficult to separate, because of the presence of isomers or other compounds that are structurally similar.

and/or their metabolites in animal tissue (e.g. blood) and (b) specific pollutants in the environment. In each of these two cases, matrix interferences are unlikely to be chemically similar to the analytes, suggesting that their separation from analyte peaks will depend strongly on T and t_G (see discussion of [1,13]).

3.2. Two-run assay procedures

One goal of the present study was the separation of complex samples by selecting different RPLC conditions for two separations (run-1 and run-2), such that every sample component is adequately resolved in one of these two runs. Specifically, we will examine the use of separations where T and t_G are optimized, as in the examples of Fig. 1. The approach will be illustrated using the 48-component sample of Fig. 2, which under the best conditions of T and t_G exhibits unacceptable resolution of the total sample (Fig. 1a, $R_s=0.4$). A later version of the software used in this study (DryLab version 3.0) allows the similar optimization of any two separation conditions (e.g., pH and gradient time, temperature and %B, etc.).

3.2.1. Option 1: sequential transfer of critical bands

One approach is to begin with all bands in run-1 of the 2-run procedure, and to sequentially designate ''hardest-to-resolve'' bands for analysis in run-2. That is, the bands designated for run-2 will eventually be determined in run-2, while remaining (undesignated) bands will be assayed in run-1. There will be fewer bands in run-2 which it is required to separate, presumably facilitating the separation of difficult bands in run-2 (rather than in run-1). ''Hardest-to-resolve'' bands are determined by their ordering in a resolution list as in Table 1; e.g., bands 42 and 43 are the first candidates for designation in this way. This procedure is illustrated in Table 3 for this same sample.

In step 1 of Table 3, T and t_G are optimized for the total sample (as in Fig. 1). The resolution of this single separation is $R_s=0.4$, which is to be improved using a 2-run procedure. In step 2, the least-resolvable band (No. 42 from Table 1) is designated for assay in run 2. The resolution of the remaining 47 compounds to be separated in run 1 is now $R_s=0.5$, while the resolution of band 42 in run 2 is $R_s=1.7$ (the determination of R_s for each run is best determined from an appropriate resolution map for

Table 3
Selecting conditions for the 2-run separation of the 48-component sample of Fig. 2 by designating critical bands in run 1 for separation in run 2. See text for details

Step	Run 1			Run 2	R_s
	Optimized conditions (run 1)	R_s	Critical band in run 1[a]	Optimized conditions (run 2[b])	
1	47°C/64 min	0.4	42		
2	68°C/22 min	0.5	43	74°C, 104 min (No. 42)	1.7
3	68°C/22 min	0.5	28	74°C, 104 min (Nos. 42, 43)	1.7
4	68°C/22 min	0.5	22	71°C, 89 min (Nos. 28, 42, 43)	1.2
5	68°C/22 min	0.5	24	34°C, 58 min (Nos. 22, 28, 42, 43)	1.1
6	68°C/22 min	0.5	27	36°C, 52 min (Nos. 22, 24, 28, 42, 43)	0.8
7	68°C/22 min	0.5	26	36°C, 52 min (Nos. 22, 24, 27, 28, 42, 43)	0.8
8	68°C/22 min	0.6	20	36°C, 52 min (Nos. 22, 24, 26–28, 42, 43)	0.8
9	68°C/22 min	0.6	9	36°C, 52 min (Nos. 20, 22, 24, 26–28, 42, 43)	0.8
10	68°C/22 min	0.6	44	36°C, 52 min (Nos. 9, 20, 22, 24, 26–28, 42, 43)	0.8
11	68°C/22 min	0.6	19	36°C, 52 min (Nos. 9, 20, 22, 24, 26–28, 42–44)	0.8
12	54°C, 56 min	0.9		34°C, 69 min (No. 9, 19, 20, 22, 24, 26–28, 42–44)	0.7

[a] E.g., band No. 42 is critical for the conditions of step 1 (Table 1); in step 2, band No. 42 has been selected for separation in run 2, so that only this compound is of interest in run 2 at this step.

[b] Bands selected for separation in run 2 indicated in parentheses.

designated compounds in that run). As long as the resolution of the designated compounds in run 2 exceeds (or equals) that in run 1, this procedure is continued as shown in Table 3. At step 12, the resolution in run 1 exceeds that in run 2, so the process has gone far enough. That is, the conditions of step 12 are preferred for a 2-run assay procedure. With these conditions, compounds 9, 19, 20, 22, 24, 26–28, 42–44 will be assayed in run 2, and the remaining 37 compounds will be determined in run 1. The critical resolution for the overall separation (runs 1 plus 2) is equal to R_s for the least resolved band pair, namely $R_s=0.7$ (in run 2).

3.2.2. Option 2: sequential transfer of critical band-pairs

An alternative procedure for the development of a 2-run method is to sequentially transfer critical band-pairs, rather than individual bands as in Table 3. This is illustrated in Table 4 for the same sample. For optimized conditions as in step 1, the critical band-pair is 42/43. Designation of this band-pair for run 2 leads to a resolution $R_s=0.5$ in run 1 and $R_s=1.7$ in run 2 (for bands 42 and 43), with a new critical band-pair in run 1: Nos. 6 and 7. In step 3, band-pairs 6/7 are also designated for run 2, and conditions for each run are optimized for the separation of compounds to be analyzed in that run (Nos. 6, 7, 42, 43 in run 2, remaining compounds in run 1). This designation of peaks for assay in run 2 is continued until the resolution in run 2 equals or is less than that in run 1, which is the case for step 6. The conditions of step 6 therefore correspond to the best overall separation of the sample for a 2-run procedure ($R_s=0.8$). Fig. 3a shows this ''best'' separation for run 1, while Fig. 3b shows the corresponding separation for run 2; (*) marks the bands to be determined in run 2. Figs. 3c and d show the corresponding resolution maps for these two separations.

The application of options 1 and 2 to several samples has shown a general advantage for option 2, as illustrated by the example of Tables 3 and 4 (overall $R_s=0.8$ for option 2 vs. 0.7 for option 1). Also, convergence to a final method proceeds more rapidly using option 2 (6 steps) vs. option 1 (11 steps). Since the computation time required for each step can be as much as a min or more, this represents another advantage of option 2. Further changes in the designation of analytes in the two runs may improve the separation of option 1 (e.g., making it equivalent to option 2), and in some cases additional changes in band designation can also improve separations resulting from option 2; however, in our experience (unreported results) the resulting improvement in overall sample resolution is usually small ($\leq$0.2 R_s-units). Software such as DryLab 2.0 makes either

Table 4
Selecting conditions for the 2-run separation of the 48-component sample of Fig. 2 by designating critical band-pairs in run 1 for separation in run 2. See text for details

Step	Run 1			Run 2	
	Optimized conditions (run 1)	R_s	Critical band in run 1[a]	Optimized) conditions (run 2[b])	R_s
1	47°C/64 min	0.4	42, 43		
2	68°C/22 min	0.5	6, 7	74°C, 104 min (Nos. 42, 43)	1.7
3	68°C/22 min	0.7	35, 36	68°C, 103 min (Nos. 6, 7, 42, 43)	1.2
4	68°C/22 min	0.7	30, 31	27°C, 70 min (Nos. 6, 7, 35, 36, 42, 43)	1.0
5	68°C/22 min	0.7	3, 5	33°C, 66 min (Nos. 6, 7, 30, 31, 35, 36, 42, 43)	0.9
6	67°C/22 min	0.8	32, 34	35°C, 61 min (No. 3, 5, 6, 7, 30, 31, 35, 36, 42, 43)	0.8
7	67°C/22 min	0.8		31°C, 72 min (No. 3, 5, 6, 7, 30, 31, 32, 34, 35, 36, 42, 43)	0.7

[a] E.g., band-pair No. 42/43 is critical for the conditions of step 1; in step 2, bands Nos. 42 and 43 are selected for separation in run 2, so that only this band-pair is of interest in run 2 at this step.

[b] Bands selected for separation in run 2 indicated in parentheses.

method development procedure (option 1 or 2) convenient, requiring no more than an hour or two by the user. This approach relies mainly on the ability to create *partial resolution maps*, similar to those of Fig. 2b; i.e., where R_s is displayed only for designated compounds in that run. No additional experiments are required for the development of 2-run assays in this way; the four initial experiments where T and t_G are varied are sufficient when using DryLab 2.0 (or later software versions).

The separations of Figs. 3a,b can be improved further, by designating the *best-resolved* peaks in each run for determination in that run. This procedure cannot improve the separation of the poorest-resolved band-pair (R_s=0.8), but the resolution of other bands may increase. This is illustrated in Table 5, where values of R_s are listed in each run for all compounds. Values of R_s in the run with best resolution of a peak are marked by (*) in Table 5, and it can be seen that a total of 28 compounds are better assayed in run 2 than in run 1 (vs. the 10 compounds designated in Fig. 3b). Moreover, many

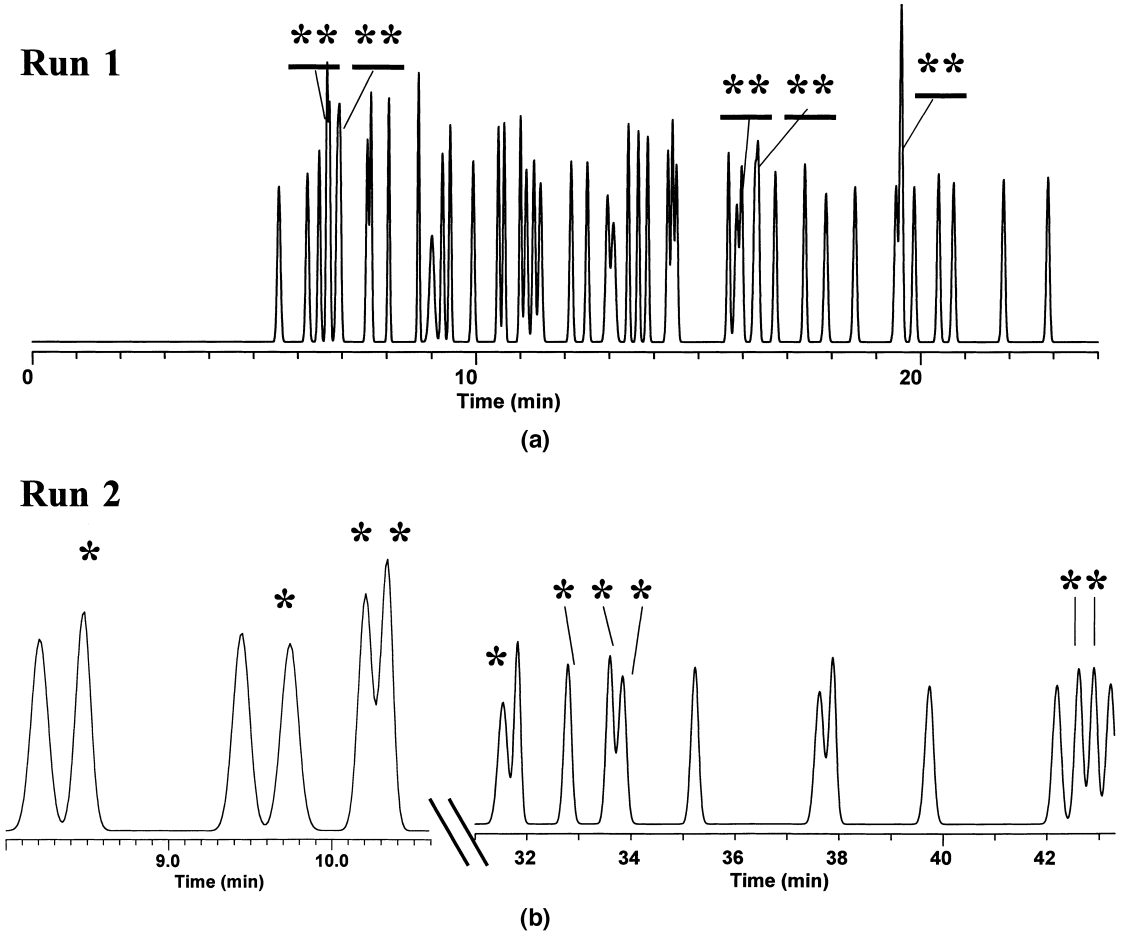
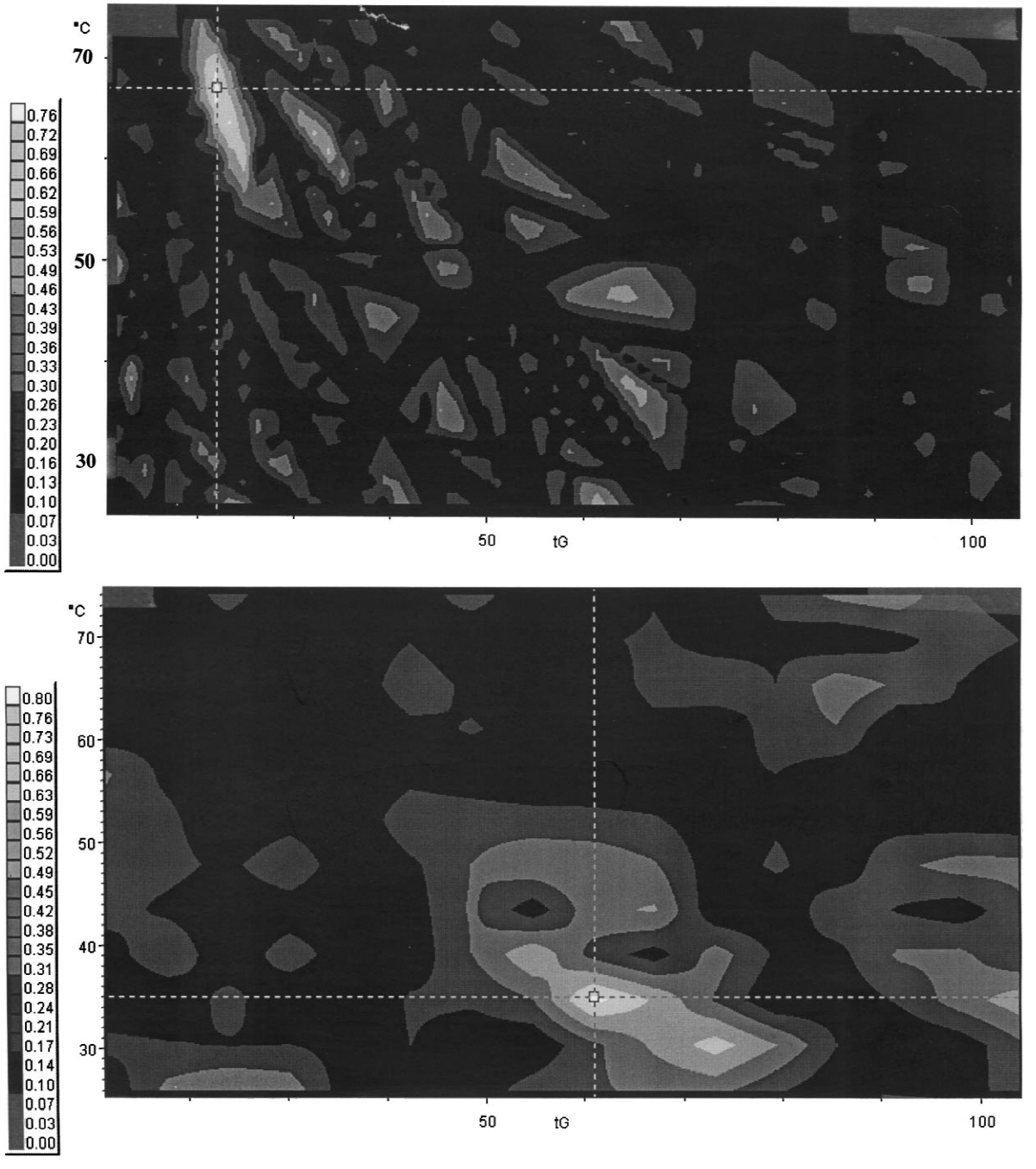


Fig. 3. Application of a 2-run assay procedure to the sample of Fig. 2 (as in Table 4). Conditions as in Fig. 1 except as follows: (a) run 1 chromatogram, T=67°C, t_G=22 min; (b) run 2 chromatogram, T=33°C, t_G=66 min (partial chromatogram showing analytes in run 2); (c) resolution map for 38 compounds to be assayed in run 1 (optimum conditions gave separation of (a)]; (d) resolution map for 10 compounds to be assayed in run 2 [optimum conditions gave separation of (b)]; (e) resolution map for run 1, where the 20 best-resolved compounds of Table 5 are designated for analysis. (*) Denotes peaks that are analyzed in run 2; remaining peaks are analyzed in run 1. (*Continued on next page*)



(c)

Fig. 3. (*continued*)

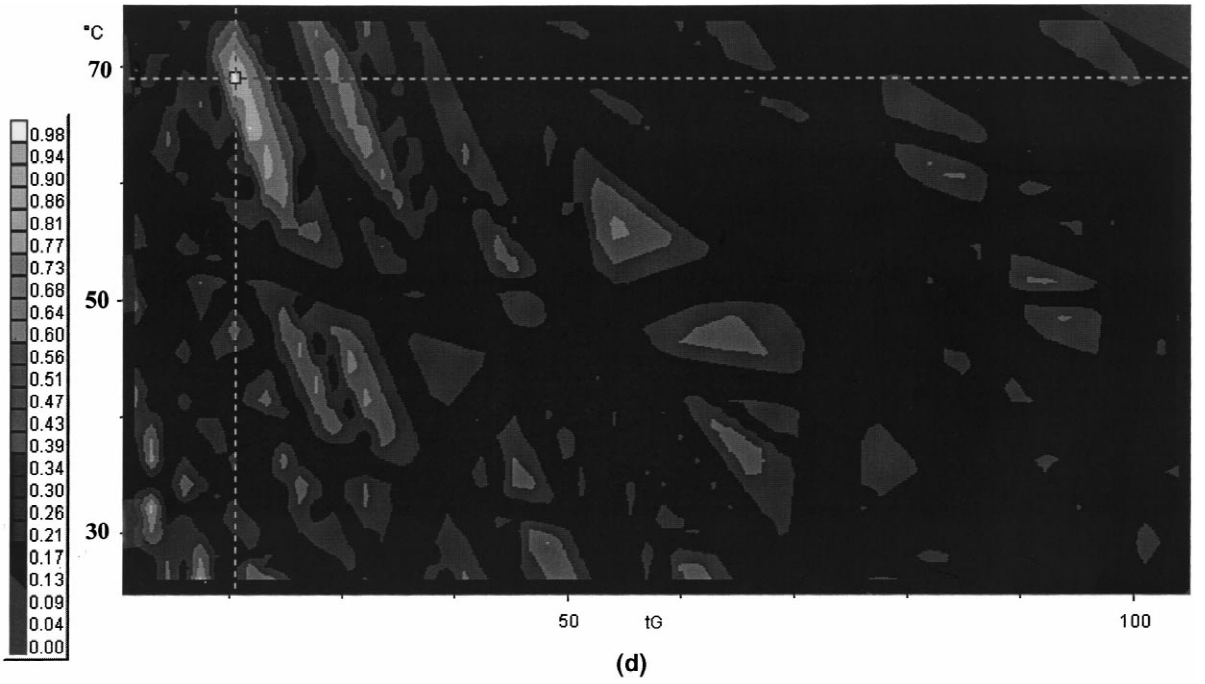


Fig. 3. (*continued*)

bands are seen to be baseline resolved ($R_s > 1.5$) in both runs.

If the 20 compounds in Table 5 that are better resolved in run 1 are designated for analysis in run 1, values of T and t_G can be re-optimized for this 20-analyte set. Fig. 3e shows the resulting resolution map, which can be compared with the map of Fig. 3c. The reduction in the total number of compounds required to be separated in run 1 allows an increase in R_s from 0.8 in Fig. 3c to 1.0 in Fig. 3e. That is, the critical designated compounds in run 1 are better separated with these new conditions of T (69°C) and t_G (20. 5 min). Re-optimization of the remaining 28 compounds in run 2 leads to the same optimum conditions and resolution as in Fig. 3d, which is necessarily the case for such 2-run procedures.

3.2.3. Application of option 2 to other samples

Two-run procedures were developed for the various samples of Table 2, excluding those where $n < 10$ – since it is more complex samples that can significantly benefit from a 2-run separation. These results are summarized in Table 6 and compared with the optimized 1-run procedure as in Fig. 2. For many samples, there is little or no increase in the attainable sample resolution for the 2-run procedure vs. the corresponding 1-run method; e.g., samples 6, 7, 9, 11. In other cases (e.g. Nos. 19, 21, 24), almost a 2-fold increase in sample resolution results for the 2-run separation. As expected, the advantage of 2-run vs. 1-run methods tends to increase for larger n. This is shown in Fig. 4, which plots the ratio of resolution for optimized 2-run ($[R_s[2]$) vs. 1-run ($[R_s[1]$) methods as a function of n. In order to minimize the (large) scatter of data in this plot, values of $[R_s(2)/R_s(1)]$ were averaged for each 5-unit interval in n (e.g. $11 \leq n \leq 15$, $16 \leq n 20$, etc.). Note also that $[R_s(2)/R_s(1)]$ must equal one when $n \leq 2$.

When R_s is marginal after optimizing T and t_G in a 1-run separation, it will rarely prove useful to develop a 2-run procedure, unless the increase in R_s is at least 30%. While Fig. 4 suggests that this will not often be the case unless $n > 15–20$, it is just these samples that are likely to be poorly separated after optimizing T and t_G for a 1-run method [1]. It should

Table 5
Resolution of each compound in the two runs of Fig. 3a,b. (*) designates highest R_s value for a solute in either run 1 or run 2

Solute	R_s in run 1	R_s in run 2
Tranylcypromine	6.7*	5.1
Amphetamine	2.2*	1.4
Acetaminophen	1.8*	1.5
N-Acetylprocainamide	0.6	0.8*
Codeine	0.6	0.8*
Methamphetamine	0.5	1.5*
Tripelennamine	0.5	1.4*
Phentermine	0.8	2.6*
β-Hydroxytheophylline	0.8	1.2*
Ethyl morphine	4.4*	2.8
Brucine	2.0	3.7*
1-Nitropropane (internal standard)	1.5*	1.2
Sulfmethanzine	1.5	2.8*
Clenbuterol	1.6*	0.8
Pythyldione	4.6*	0.8
Chlordiazepoxide	1.3	4.0*
Vincamine	1.3	4.0*
Doxepram	1.2*	0.4
Indole-3-carboxaldehyde	1.2*	0.4
Apobarbital	1.1	1.2*
Salicylic acid	1.1	2.0*
Butabarbital	3.0*	0.4
Butethal	3.0*	0.1
Acetophenone	0.8*	0.1
1-Nitrobutane (internal standard)	0.8*	0.4
Desipramine	2.2*	1.2
Imipramine	2.0*	1.4
Cortisone	2.0*	1.2
Oxazepam	0.9	2.4*
Chlorpromazine	0.8	1.8*
2-Naphthoxyacetic acid	0.8	1.8*
fluoxymesterone	1.3*	0.9
1-Nitropentane (internal standard)	0.8	*.9
Flunitrazepam	0.8	3.0*
Chloroxylenol	0.6	0.9*
Lometazepam	0.6	0.9*
Butylparaben	3.0	4.9*
Diflunisal	3.5*	0.9
1-Nitrohexane (internal standard)	3.5*	0.9
Danthron	4.5	6.4*
1-Nitroheptane (internal standard)	0.8	1.4*
Phenylbutazone	0.0	1.0*
Mefenamic acid	0.0	1.0*
Biphenyl	2.1*	1.1
Danazol	2.5	3.4*
1-Nitrooctane (internal standard)	2.5	3.4*
1-Nitrononane (internal standard)	7.4	9.6*
1-Nitrodecane (internal standard)	7.4	9.6*

also be noted that a plot of $[R_s(2)/R_s(1)]$ vs. n for individual samples (not shown in Fig. 4) is markedly scattered (see Table 6), suggesting that the advantage of a 2-run procedure for a given sample can be considerably greater than suggested by the averaged data of Fig. 4.

3.2.3.1. Advantages and disadvantages of 2-run methods

The increased resolution that may result from a 2-run procedure is an obvious advantage. Another attractive feature of such methods is that they can be quickly developed and evaluated (by computer), following the completion of an optimized 1-run procedure. Thus, the development of 2-run assay procedures in this way requires only four experimental runs (two different values of T and t_G), following which computer simulation (DryLab 2.0) can provide optimum values of T and t_G as illustrated in Fig. 1a. An additional 1–2 h of computer simulation with the same input runs can then lead to an improved 2-run procedure, as illustrated in Fig. 3 and Tables 4 and 5.

There are also some significant disadvantages to the 2-run approach. First, two RP-LC runs are required in place of a single run, usually representing a doubling of the time and effort required for the analysis of each sample. A second consideration is the need to combine results from two separate runs into a single final sample analysis. The bookkeeping requirements are easily dealt with in the modern, computerized laboratory, but small variations in either sample injection volume or detector sensitivity between runs 1 and 2 must be corrected for. This is readily accomplished by using one or more well resolved peaks in each run to normalize response factors for the two runs. For the two separations of the present sample as in Table 5, there are several compounds that might be used in this way; e.g., ethylmorphine, danthron, danazol, and nitro-C_8, -C_9, or -C_{10}.

Finally, 2-run methods appear most attractive for the case of complex samples where $n>15$–20. Our experience is that both 1-run and 2-run methods for such samples tend to be sensitive to small changes in T or t_G, implying that method transfer and robustness can be problematic. Hence, both 1- and 2-run RP-LC methods for complex samples will generally have

Table 6
Summary of 2-run methods developed for the samples of Table 2, with comparison of overall sample resolution for 1-run vs. 2-run procedures

Sample		Conditions	*n*	1-Run R_s	2-Run R_s
6	Synthetic organics		11	2.6	2.6
7	Algal pigments	col-A	12	3.3	3.3
7		col-B	12	1.3	1.3
8	Herbicides		13	1.6	1.8
9	LSD derivatives		13	0.8	0.8
10	Fatty acid esters	ACN, col-B	13	0.3	0.3
10		MeOH, col-B	14	0.6	0.6
10		ACN, col-A	14	0.9	1.1
11	Acrylate monomers	ACN	14	1.4	1.4
11		MeOH	14	1.3	1.3
12	Benzoic acids+anilines	pH 2.6	14	1.3	2.0
13	Basic drugs		15	1.9	2.5
14	Testosterones		17	0.2	0.3
15	Herbicides	pH 2.7	19	0.6	0.7
		pH 3.5	19	0.7	1.0
16	rhGH digest		20	3.0	3.3
17	Synthetic organics	column 1, ACN	21	0.5	0.6
		column 2, ACN	21	0.7	0.7
		column 1, MeOH	22	1.1	1.2
18	Nonbasic drugs		25	1.1	1.8
19	Algal pigments		29	0.7	1.4
20	Synthetic organics		33	0.5	0.5
21	rtPA digest		37	0.5	0.9
22	Basic+nonbasic drugs		40	0.7	0.9
23	drug sample	ACN	47	0.6	0.6
		MeOH	47	0.4	0.6
24	No. 22 toxicology+nitroalkane standards		48	0.4	0.8

less tolerance for marginal equipment or laboratory practice. Another consideration which becomes important for complex samples is that peak tracking is required for each of the four initial experiments that precede computer simulation, and frequent peak overlaps (as in Fig. 1 of Part III [14]) can make retention time measurements less reliable. These problems (which are especially important for complex samples) can be reduced by the use of selective detection (UV diode-array, MS), by carrying out additional experimental runs, or (where applicable) by carrying out additional separations with known standards.

It should be kept in mind that an increase in N (as by increasing column length, reducing flow-rate or particle size, or using capillary electrochromatography) represent alternatives to the use of 2-run methods. It is likely that this latter option will be preferable for some samples, but not for others. Computer simulation can also be used to compare the relative advantages of one approach vs. another, again with little investment of time. Yet another

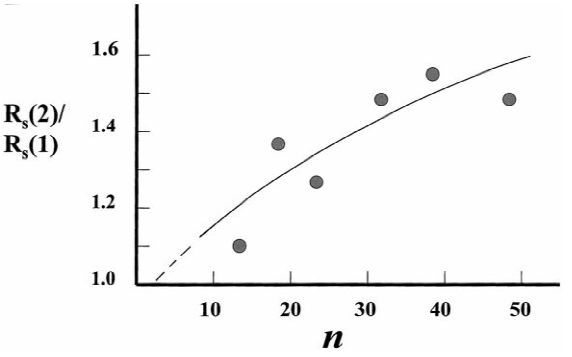


Fig. 4. Improvement in resolution for 2-run procedures (R_s[2]) vs. 1-run procedures (R_s[1]) as a function of the number n components in the sample. Based on data of Table 5, with averaging of data for similar values of n. The curve through these data is a subjective fit.

alternative to the use of 2-run assay procedures is the use of selective detection (UV–diode-array, MS) for separations that do not provide adequate resolution of all peaks.

It is possible to extend the concept of 2-run assay procedures to the use of three or more runs where T and t_G are allowed to vary. For the 48-component sample used as example in Tables 1, 3 and 4, this would allow total sample resolution to approach $R_s=1.7$ for a large enough increase in the number of assay runs. However, this multi-run approach appears impractical for most samples.

3.3. Hard-to-resolve band-pairs

For most samples, the data of Table 2 suggest that individual compounds can be easily resolved from the remainder of the sample, using RP-LC with optimization of T and t_G. The resolution of the total sample using a 1-run (or 2-run) procedure is thus limited by both sample type and the value of n [1]. There are exceptions to this generalization, however, and such exceptions may in turn limit our ability to separate the total sample. Thus, referring to the resolution list of Table 1, it is clear that the maximum possible resolution cannot exceed $R_s=1.7$ for this sample, because this is the best possible resolution of bands 42 or 43 from remaining peaks. This is true for procedures based on one run, two runs, or any number of runs.

In many cases, the maximum possible resolution with either a 1- or 2-run procedure is limited by difficulty in separating one or more hard-to-resolve pairs of compounds in the sample. That is, the resolution of such band-pairs from each other is not strongly dependent on T or t_G. In most cases, hard-to-resolve band-pairs are expected to consist of structurally similar molecules, especially isomers. Thus, the Martin equation [12] predicts equal values of k for each of two isomers, and similar values of k for structurally related compounds. Similarly, it has been shown [13] that changes in selectivity (α) for two adjacent bands as T or t_G is changed will be larger, the more different are the two molecules in terms of polar substitution.

Sample 5 of Table 2, which consists of a mixture of corticosteroids, provides an interesting illustration of the role of molecular similarity in limiting maximum sample resolution. Fig. 5 shows the optimized separation of the total sample, and Table 7 summarizes differences in molecular structure for adjacent band-pairs from Fig. 5. For example, compound 1 contains an 11-OH group, which in band 2 is replaced by a carbonyl oxygen. Bands in group-B of Fig. 5a differ from those in group-A by the presence of an additional methyl and fluoro group in compounds 7 and 8 (band 9 is of no interest in the present discussion). Also shown in Table 7 are maximum R_s values for the separation of (a) individual compounds from the total sample (next to last column) and (b) certain band-pairs (those adjacent in Fig. 5) from each other (last column).

The separation of band-pairs 1/2 and 7/8 in the total sample is seen to be limited by their separation from each other; i.e., the band and band-pair R_s values are equal for each of these band-pairs. The separation of bands 3–6 from the rest of the sample is limited mainly by the crowding of these bands into a small region of the chromatogram (although this reflects their structural similarity). Interestingly, the separation of various isomeric band-pairs Nos. 2/3 ($R_s=10.7$), Nos. 3/5 ($R_s=4.2$) and Nos. 7,8 ($R_s=2.3$) is on average better than that of adjacent *non*-isomeric bands which differ only in the presence of an -OH vs.=O substituent at position 11: Nos. 1/2 ($R_s=1.4$), Nos. 3/4 ($R_s=2.0$), Nos. 4/5 ($R_s=3.8$), Nos. 5/6 ($R_s=2.0$).

In the past, the presence of isomers in a sample

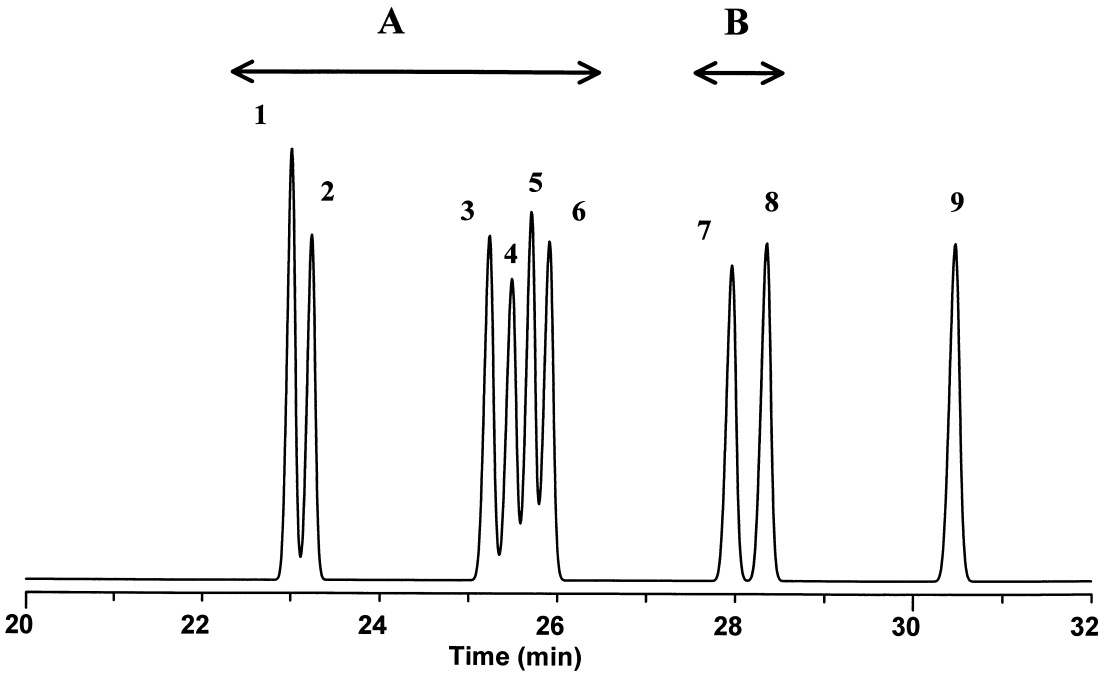


Fig. 5. Optimized separation of entire corticosteroids sample (see Table 7) and individual band-pairs (T=29°C, t_G=54 min).

has often led chromatographers to anticipate difficulty in their RP-LC separation. The example of Table 7 suggests that this may not necessarily be so, when T and t_G are optimized for a given pair of isomers. Similarly, both samples 1 and 2 of Table 2 contain ionizable isomers whose separation by varying T and

Table 7
Optimized RP-LC separation of individual compounds from total sample and adjacent band-pairs from each other for corticosteroid sample (No. 5 of Table 2); T and t_G optimized in each case, other conditions as described previously [11]

Group[a]	Compound	Structure change (band $i \rightarrow i+1$)[b]	R_s for band[c]	R_s for band-pair[d]
A	(1) 20-Dihydroprednisolone	11-OH→11-C=O	1.4	1.4
	(2) 20-Dihydroprednisone	Interchange of –OH and –C=O at 11 and 20	1.4	10.7
	(3) Hydrocortisone	11-OH→11-C=O	1.8	2.0
	(4) Prednisolone	11-C=O→11-OH	1.1	3.8
	(5) Prednisone	11-OH→11-C=O, C=C→CH–CH	1.3	2.0
	(6) Cortisone		1.7	12.4
B	(7) Betamethasone	Stereoisomers	2.3	2.3
	(8) Dexamethasone	Not relevant (last band ignored)	2.3	11.2
	11-Deoxycortisol		11.2	

[a] See Fig. 5.
[b] E.g., for compound No. 2 vs. compound No. 1, an -OH group replaces a =O group at position 11.
[c] The maximum R_s for the compound from all other sample compounds, when T and t_G are optimized.
[d] The maximum resolution of compound 1 from 2 (R_s=1.4), 2 from 3 (R_s=10.7), etc.

t_G is remarkably easy (maximum $R_s>10$), possibly the result of well-known changes in pK_a as a result of change in T or t_G (equivalent to change in %B). Work is in progress (J.W.D., L.R.S.) to further explore the separation of isomers by varying T and t_G.

4. Conclusions

The present study has examined our ability to separate one or more compounds from samples containing as many as 48 components, using variation in temperature T and gradient time t_G to optimize their RPLC separation. With few exceptions (mainly isomers), it was possible to separate individual compounds from these various samples with a resolution $R_s>1$. The adequate resolution of all sample components in a single separation is more difficult, especially when the number n of such components exceeds 15–20. For $n>20$, it is possible to increase total-sample resolution by an average of 30–50%, (but sometimes more) by using two different runs where T and t_G have been separately optimized for different sample components. As a result, samples which cannot be adequately separated in the conventional manner (single assay run) may be separable using a 2-run assay procedure.

Two possible means of developing a 2-run assay procedure were investigated, based on the use of computer simulation (DryLab) and successively designating either bands or band-pairs to be determined in run 2. Of these two approaches, the successive designation of critical band-pairs was found more effective. The development of a 2-run method in this way requires little effort and no additional experimental data, assuming that four experiments (with T and t_G varying) are carried out first as a means of developing an optimized 1-run method.

The present study (see Table 2) combined with other observations [13] suggests that the separation (and analysis) of one or a few compounds from structurally unrelated compounds in complex mixtures generally will be successful, when temperature and gradient time are optimized. Common examples of this kind include the determination of drugs and/or their metabolites in plant or animal tissue, as well as various environmental assays. Because of other advantages of this approach to RPLC method development [13,15], its use for applications of this kind can be recommended.

5. Symbols

See Glossary of terms section in Part I [1].

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