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BAND-SPACING IN REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AS A FUNCTION OF SOLVENT STRENGTH

A SIMPLE AND FAST ALTERNATIVE TO SOLVENT OPTIMIZATION FOR METHOD DEVELOPMENT

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SUMMARY

Numerous reports have described the use of solvent optimization for isocratic reversed-phase high-performance liquid chromatography method development. Solvent optimization involves the use of different solvents (usually methanol, acetonitrile and tetrahydrofuran) to control band-spacing for maximum resolution of the sample. Here, we examine an alternative approach, based on variation of the concentration of organic solvent in the mobile phase (solvent strength). This procedure is less powerful than classical solvent optimization, but it nevertheless possesses a significant ability to effect changes in band-spacing. It is also much more easily carried out. Many samples do not require solvent optimization, and in these cases, a change in solvent strength may be the more practical approach.

The retention data required for solvent-strength optimization are most conveniently collected by using two initial gradient runs. The application of gradient retention data for developing a final isocratic separation is facilitated by the use of commercial software. The advantages and limitations of gradient-retention data for this purpose are examined.

INTRODUCTION

A major part of method development in high-performance liquid chromatography (HPLC) consists of achieving satisfactory resolution of the sample. The basic equation for resolution, R_s, in isocratic HPLC[1],

$$R_s = (1/4)\,(\alpha - 1)\,N^{0.5}\,[k'/(1 + k')] \qquad (1)$$

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tells us that separation is affected by solvent strength (capacity factor k'), separation factor α, and column plate number N. Usually method development begins with adjustment of the mobile phase solvent strength [percent water in reversed-phase (RP) HPLC] to achieve adequate values of k', followed by change in conditions to optimize band-spacing (values of α for adjacent bands). Finally, an increase in N can be obtained by varying "column conditions": column length, particle size and flow-rate[2]. Conditions for satisfactory values of k' and N are usually not difficult to find, so that the main challenge is in optimizing α values.

Several studies have been reported for RP-HPLC, that show large changes in band-spacing as a result of varying the organic solvent(s) used in the mobile phase[3–8]. This has led to a more or less standard approach to mobile phase optimization for isocratic RP-HPLC: mapping sample resolution as a function of the composition of mobile phases that are prepared from mixtures of methanol, acetonitrile, tetrahydrofuran (THF) and water[6]. The result has been accurately described as "automated method development"[8]. However, such an approach often requires a large number of experimental runs to adequately map resolution as a function of mobile-phase composition; *e.g.*, seven runs for the sample and for each sample component. Specialized software is also needed to get the most out of this approach.

An alternative is to make use of some other change in separation conditions to alter band-spacing; *e.g.*, bonded-phase or column type[9,10], temperature[11], or solvent strength (mobile phase water content)[5,12]. Gant *et al.*[13] examined the combined effect of changes in solvent strength and temperature, so as to maintain analysis time approximately constant (see also the similar studies of Melander *et al.*[14]). In this paper we examine the variation of solvent strength as a means of changing band spacing and optimizing retention. Here and elsewhere[15] we also describe an alternative approach to optimizing band-spacing, based on initial gradient runs plus variations of solvent strength. A procedure based on the same principle has been described for the gradient-elution separation of peptide samples[16].

EXPERIMENTAL

Steroid separations

Equipment. The HPLC system was a DuPont 8800 liquid chromatograph (DuPont, Wilmington, DE, U.S.A.) equipped with a Model 860 fixed-wavelength detector and heated column compartment. Gradient simulations and calculations were carried out with DryLab™ 45 software from LC Resources Inc. (San Jose, CA, U.S.A.), using an IBM XT personal computer.

Reagents. Solvents were HPLC-grade acetonitrile and THF (J. T. Baker, Phillipsburg, NJ, U.S.A.). A Milli-Q system with Organex-Q cartridge (Millipore, Bedford, MA, U.S.A.) was used for water purification.

Samples. Steroid samples were obtained from Roussel Corp. (New York, NY, U.S.A.) and were of pharmaceutical grade.

Nitroaromatic separations

Equipment. An IBM (Danbury, CT, U.S.A.) Model 9533 HPLC system was used; columns were placed in a closed column compartment and operated at ambient temperature. An IBM Model 9522 fixed-wavelength UV detector was used at 254

nm. Data were collected with an IBM Model 9000 data system with CAP 1.4 software.

Reagents. HPLC-grade methanol was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.) and water was purified in a Nanopure II purification unit (Barnstead Co., Boston, MA, U.S.A.). Before use, all solvents were filtered through a 0.45-μm filter and vacuum-degassed; continuous helium sparging was used to maintain degassed solvents air-free during HPLC operation.

Samples. All nitroaromatic samples were of reagent grade or better, obtained from J. T. Baker and City Chemical Corp. (New York, NY, U.S.A.).

Software. The DryLab 45 software used in this work is available from LC Resources Inc., 1933 Adele Place, San Jose, CA 95125, U.S.A.

RESULTS AND DISCUSSION

Band spacing and solvent strength

In RP-HPLC, retention can usually be described by the approximate (empirical) relationship

$$\log k' = \log k_w - S\varphi \tag{2}$$

where φ is the volume fraction of organic solvent in the mobile phase, k_w is the (extrapolated) value of k' for water as mobile phase ($\varphi = 0$), and S is a constant characteristic of the given compound. Eqn. 2 is normally adequately accurate over the k' range of usual interest ($1 < k' < 10$). An example of the validity of eqn. 2 is shown in Fig. 1, where retention data are plotted *vs.* φ for a series of compounds

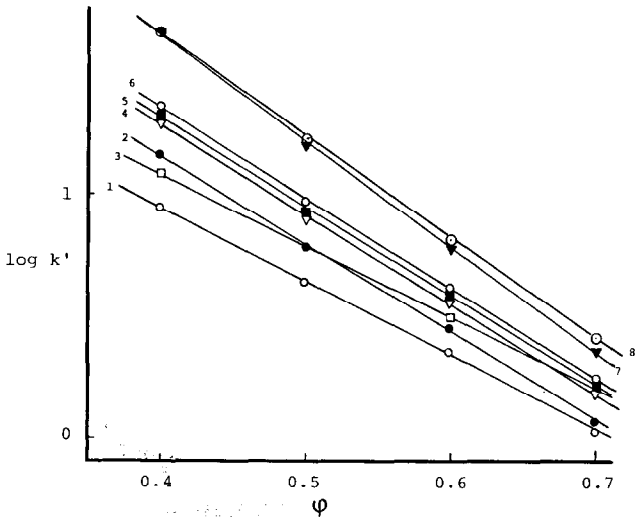

Fig. 1. Plots of log k' *vs.* volume fraction of organic solvent (φ) for a mixture of nitroaromatics. See Table III for details.

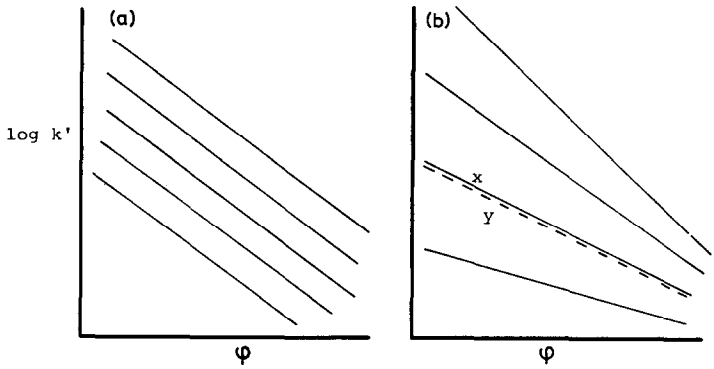

Fig. 2. Hypothetical plots of log k' *vs.* φ for related solutes, where variation in φ does not improve the separation of closely adjacent bands. (a) All S values equal. (b) S values increase with solute retention; x and y refer to bands that overlap.

(methanol–water, C_8 column). For each of these eight solutes, the data points fall reasonably close to a straight line*.

When the slopes S of two solutes are equal (parallel plots of log k' *vs.* φ), the separation factor α for the two compounds is not a function of solvent strength (value of φ). This is the case for compounds 5 and 6 in Fig. 1, so that their relative band-spacing cannot be altered by changes in φ. In the case of bands 2 and 3 of Fig. 1, on the other hand, the S values differ, and a change in φ leads to a change in relative retention. This in turn means that the resolution of these two bands will vary markedly as solvent strength φ is varied; *e.g.*, $R_s = 0$ at $\varphi = 0.52$ (methanol–water, 52:48).

Our ability to improve band spacing by changes in φ depends on the S values of the sample compounds. There are two cases where a change in φ will not change band spacing: (a) when all the solute S values are equal (or nearly so), as in Fig. 2a; and (b) when there is a strong correlation of solute S values with corresponding k' values, as in Fig. 2b. Note in Fig. 2b that a decrease in φ for this case can improve separation by increasing α values for all adjacent solute-pairs. However, if two bands are essentially unresolved at one value of φ (*e.g.*.., bands x and y of Fig. 2b) they will be unresolved at other values of φ. Because closely adjacent (overlapping) bands present the major challenge in method development, S values as in Fig. 2b will generally preclude any real improvement in band spacing by varying φ.

It is known[18] that values of S generally increase with the molecular size of solutes. Therefore, if two compounds differ significantly in molecular weight and are unresolved for a given value of φ, a change in φ should result in their resolution. The further dependence of S on other aspects of solute structure is still unclear. The present study was undertaken, in part, to clarify this question.

Studies of Schoenmakers et al. Schoenmakers *et al.*[5] reported S values for 32 small-molecule solutes of varied structure. The main conclusions drawn in their paper with regard to S values as a function of the solute were as follows:

(1) For methanol as organic solvent, values of S correlate strongly with log k_w (correlation coefficient, $r = 0.98$), as in Fig. 2b.

* The following discussion is also applicable for cases where these plots have a slight curvature[17].

(2) For THF as organic solvent, there is a weak correlation between S and log k_w ($r = 0.76$).

(3) For acetonitrile as organic solvent, there is no significant correlation between S and log k_w ($r = -0.06$).

These results would lead us to conclude that acetonitrile–water mobile phases will give significant changes in band spacing as φ is varied, while methanol–water mobile phases will show little change in band-spacing as a function of φ (THF–water mobile phases are intermediate). However, the actual situation is more complicated.

We have carried out a detailed examination of values of S *vs.* solute structure (using the raw data from ref. 5). As noted above, variations in S that are a function of sample retention (as in Fig. 2b) are of little value in changing band-spacing (for the case of $\alpha \approx 1.00$). Therefore, we first eliminated contributions to S that were retention-dependent. The correlations between S and retention, reported by Schoenmakers *et al.*[5], are as follows (base-10 logarithms, rather than the natural logarithms reported in ref. 5):

$$\text{(Methanol)} \quad S = 0.99 + 0.34 \log k_w \tag{3}$$

$$\text{(THF)} \quad S = 1.88 + 0.34 \log k_w \tag{4}$$

$$\text{(Acetonitrile)} \quad S = 2.55 \tag{5}$$

The retention-independent contribution to S can be defined as the experimental S value minus the value calculated from eqns. 3, 4 or 5, respectively. These residual ΔS values then determine the potential change in band spacing that might result when φ is varied, for a band pair that is unresolved for some value of φ. That is, if two compounds have different values of ΔS for a given organic solvent, then a change in φ should yield values of $\alpha \neq 1.00$ for some value of φ.

An examination of ΔS values, determined from the data in ref. 5, shows many apparent regularities, and it appears that values of ΔS correlate strongly with the functionality of the solute. This is illustrated in Table I for four solute classes with methanol as solvent: alkylbenzenes, alkylphenols, phenylalkanols, and dialkyl phthalates. In this case, ΔS values for alkylbenzenes are about 0.5 units smaller than are ΔS values for dialkyl phthalates having similar retention (similar k' values). Differences in ΔS of this magnitude can lead to useful changes in band spacing when φ is varied (for compounds having similar retention)*.

Table II summarizes data, as in Table I, for other solutes and mobile phases from ref. 5. Here, average values of ΔS are reported for solutes of similar functionality, along with the variation (1 S.D.) in this quantity. These results are useful for anticipating when band-spacing changes will result from variation in φ. Thus, large differences in ΔS values for different solutes and a given organic solvent suggest the use of that solvent for band-spacing optimization via changing φ. On this basis, THF

* It should be noted that the correlations of eqns. 3–5 are empirical, as is the approximate constancy of S values for solutes of similar functionality. We also do not mean to imply that "retention-dependent" and "retention-independent" contributions to S reflect different retention processes or "mechanisms". Our discussion here is purely functional and is aimed only at the practical question of how separation will change as mobile phase composition is varied by changing φ.

TABLE I

SIMILARITY OF S VALUES FOR SOLUTES OF SIMILAR FUNCTIONALITY WITH METHANOL–WATER AS THE MOBILE PHASE

Based on experimental S values in ref. 5. ΔS values were calculated as experimental S value minus value of eqn. 3.

Compound	ΔS	*Compound*	ΔS
Alkylbenzenes		*Alkylphenols*	
Benzene	−0.28	Phenol	0.06
Toluene	−0.22	*o*-Cresol	0.01
Ethylbenzene	−0.17	2,4-Dimethylphenol	−0.03
		Quinolone	0.03
		m-Nitrophenol	0.03
Average	−0.23 ± 0.05 (1 S.D.)		0.02 ± 0.03
Phenylalkanols		*Dialkyl phthalates*	
Benzyl alcohol	0.09	Dimethyl phthalate	0.40
1-Phenylethanol	0.08	Diethyl phthalate	0.26
2-Phenylpropanol	0.07		
3-Phenylpropanol	0.06		
Average	0.07 ± 0.01		0.33 ± 0.10

and acetonitrile appear somewhat more promising than methanol, but considerable band-spacing control should be possible with methanol as well. This is illustrated in the example of Fig. 1 (methanol–water mobile phase), where several changes in band spacing are seen as φ is varied.

TABLE II

VARIATION IN S VALUES WITH SOLUTE TYPE AND MOBILE PHASE

Data from ref. 5 treated as in Table I.

Solute type	*n*	ΔS^*		
		Methanol	*THF*	*Acetonitrile*
Alkylanilines	3	−0.1 ± 0.1	−0.4 ± 0.1	−0.4 ± 0.2
Alkylbenzenes	3	−0.2 ± 0.0	−0.3 ± 0.0	−0.2 ± 0.2
Chlorobenzene	1	−0.2	−0.2	−0.1
Benzaldehyde, phenyl ketones	2	0.0 ± 0.1	−0.1 ± 0.1	−0.2 ± 0.1
Phenylalkyl ethers	2	−0.2**	−0.2 ± 0.0	−0.1 ± 0.1
Polyaromatic hydrocarbons	2	0.2 ± 0.1	0.0 ± 0.0	−0.1 ± 0.2
Biphenyl	1	−0.1	−0.1	0.1
Benzonitrile	1	0.1	−0.1	−0.2
Nitrobenzenes	2	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.1
Alkyl phthalates	2	0.3 ± 0.1	0.3 ± 0.0	0.2 ± 0.0
Phenylalkanols	4	0.1 ± 0.0	0.2 ± 0.2	0.4 ± 0.1

* Values for mobile phases containing indicated organic solvents.

** One obviously incorrect value discarded.

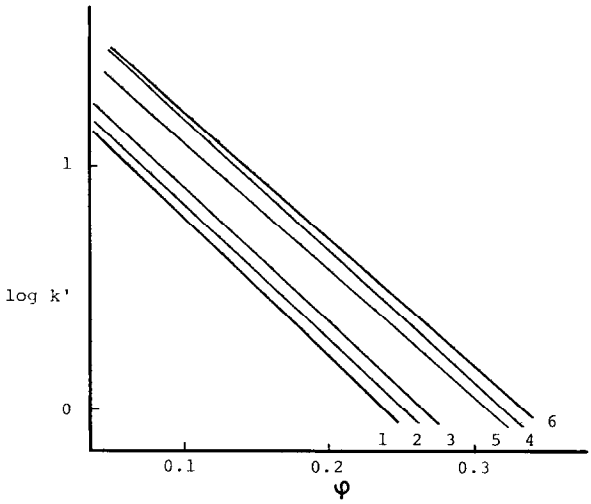

Fig. 3. Plots of log k' *vs.* φ for steroid mixture. See Table VI for details.

The data of Table II can also be used to suggest useful differences in ΔS for a given sample. The sample in Fig. 1 is a mixture of nitroaromatics plus benzene, separated with methanol–water mobile phases. Benzene (Table II) is in the alkylbenzene group, with $\Delta S = -0.2$. Nitrobenzenes have $\Delta S = +0.2$ for this mobile phase. Therefore the S value for benzene should be about 0.4 units smaller than for adjacent nitroaromatics in this sample. Compound 3 in Fig. 1 is benzene, and its S value (slope of log k' *vs.* φ) is seen to be significantly lower than surrounding solutes (nitrobenzene derivatives), as predicted from the difference in ΔS values. Note also that the S values of adjacent nitroaromatics in Fig. 1 (all bands except No. 3) are fairly similar, as suggested by the data in Table II. However, even for the nitroaromatics we see that isomeric compounds 7 and 8 are unresolved when the value of φ is low enough ($\varphi = 0.4$), reflecting a difference in their S-values.

Compounds of similar molecular weight and functionality

Molecules having similar size and functionality are expected to have similar S values. This is, in fact, the case, as the retention data in Fig. 3 illustrate. Here, plots of log k' *vs.* φ are shown for six steroids discussed in a following section. The molecular weights of these compounds fall within a $\pm 5\%$ range, and the functional groups are hydroxyls and carbonyls for each compound. It is seen that the slopes of these plots in Fig. 3 are all similar, with no change in band position as φ is varied. However, it would be a mistake to conclude that φ variation cannot be useful for this sample, as we will shortly see.

Use of gradient elution to optimize solvent strength and band-spacing for corresponding isocratic separations

We believe that the use of gradient elution for initial method development is preferable to the use of corresponding isocratic elution for several reasons:

(1) For unknown samples, a gradient experiment is more likely to reveal bands that might otherwise be lost in the solvent front (eluted at column dead-time, t_0) or

TABLE III

RETENTION DATA FOR NITROAROMATIC MIXTURE

Conditions: two 8 × 0.4 cm, 5-μm Reliance® cartridges* in series; methanol–water mixtures as mobile phase; flow-rate, 0.7 ml/min; temperature, 25°C. Compounds: 1 = nitrobenzene; 2 = 2,6-dinitroluene; 3 = benzene; 4 = 2-nitrotoluene; 5 = 4-nitrotoluene; 6 = 3-nitrotoluene; 7 = 2-nitro-1,3-xylene; 8 = 4-nitro-1,3-xylene. Column dead-time t_0 = 1.84 min; gradient dwell-time t_D = 8.14 min.

Solute	*Retention time (min)*						
	*Gradient runs***			*Isocratic runs*§			
	20 min	*40 min*	*80 min*	*40%*	*50%*	*60%*	*70%*
1	22.77	31.47	44.26	18.30	9.84	5.95	3.77
2	23.76***	34.02	50.80	28.48	13.12***	7.01	4.01
3	23.76***	33.17	47.08	24.05	13.12***	7.64	4.62***
4	24.48	35.42	53.80	37.58	16.52	8.40	4.62***
5	24.66	35.79	54.50	40.06	17.58	8.87	4.83
6	24.90	36.25	55.41	43.84	19.08	9.48	5.08
7	25.80	38.45	60.73	88.00	31.16	12.82	5.99
8	26.08	38.87	61.28	88.00	32.51	13.88	6.55

* DuPont.
** For different gradient times t_G.
*** Overlapping bands.
§ For different percentages methanol in methanol–water mobile phases.

disappear as late-eluted bands; gradient elution is also more likely to provide initial separation of bands that are clustered at the front of the chromatogram.

(2) Only two gradient experiments are required to define completely the separation characteristics of a sample with a broad k' range (for a given combination of mobile phase solvents); several isocratic runs would typically be required to obtain the same information.

(3) Some samples require gradient elution for effective separation, others can be resolved isocratically; gradient elution provides information that can be used for either type of sample.

We will illustrate this approach for the nitrocompounds in Fig. 1.

Relative accuracy of isocratic data predicted from gradient data. Any two isocratic experiments, carried out with different values of φ, can be used to determine values of S and k_w from eqn. 1. This then allows prediction of retention for any value of φ, using eqn. 1. We have previously described[17] the similar derivation of S and k_w values from two gradient experiments with different gradient times, t_G, and have shown that reliable and precise values of k' *vs.* φ can also be obtained in this way. The present study permits a further examination of this approach, because the data in Table III allow us to compare values of S and log k_w derived from either isocratic or gradient experiments. This comparison is summarized in Table IV.

First, consider derived values of S and k_w. Table IV gives the best values of S and log k_w for each compound in Table III, as derived from best-fit curves to the isocratic data of Fig. 1. We have used various pairs of runs (isocratic and gradient) from Table III to derive corresponding values of S and log k_w for comparison with

TABLE IV

COMPARISON OF ISOCRATIC PARAMETERS S AND LOG k_w

Derived from either gradient or isocratic runs in Table III. Gradient run pairs: 20/40 min, 40/80 min and 20/80 min refer to different pairs of gradient runs (20/40 min refers to calculations from 20-min and 40-min runs). Isocratic run pairs: 40/60%, etc. refer to calculations for two isocratic runs with 40 and 60% methanol in water.

Solute (see Table III)	*Value of parameter calculated in different ways**						
	Gradient run pairs			*Isocratic run pairs*			*Best***
	20/40 min	*40/80 min*	*20/80 min*	*40/60%*	*50/70%*	*40/70%*	
	S-values						
1	3.20	2.90	3.05	3.01	3.09	3.10	3.10
2	3.77	3.62	3.70	3.56	3.58	3.63	3.58
3	2.99	2.73	2.86	2.92	3.04	3.01	2.95
4	3.73	3.75	3.74	3.68	3.61	3.70	3.67
5	3.74	3.73	3.73	3.68	3.61	3.69	3.68
6	3.72	3.73	3.72	3.70	3.63	3.71	3.72
7	4.20	4.30	4.25	4.47	4.25	4.39	4.20
8	4.03	4.11	4.07	4.27	4.07	4.21	4.03
Average error	0.08	0.07	0.06	0.06	0.05	0.04	–
Average change in k'***	2×	2×	4×	5×	5×	11×	
	log k_w values						
1	2.21	2.09	2.14	2.16	2.18	2.19	2.19
2	2.25	2.13	2.18	2.25	2.31	2.29	2.26
3	2.67	2.60	2.63	2.59	2.58	2.61	2.59
4	2.77	2.78	2.78	2.76	2.71	2.77	2.76
5	2.81	2.80	2.80	2.79	2.74	2.84	2.85
6	2.84	2.84	2.84	2.84	2.79	2.84	2.85
7	3.30	3.36	3.34	3.46	3.33	3.43	3.33
8	3.24	3.29	3.26	3.38	3.26	3.35	3.27
Average error	0.03	0.04	0.04	0.04	0.03	0.03	–

* Isocratic parameters calculated from eqn. 2, using two runs of different methanol–water compositions.

** Parameters derived from best-fit curves in Fig. 1.

*** Average change in solute k' values for the two runs used to measure S.

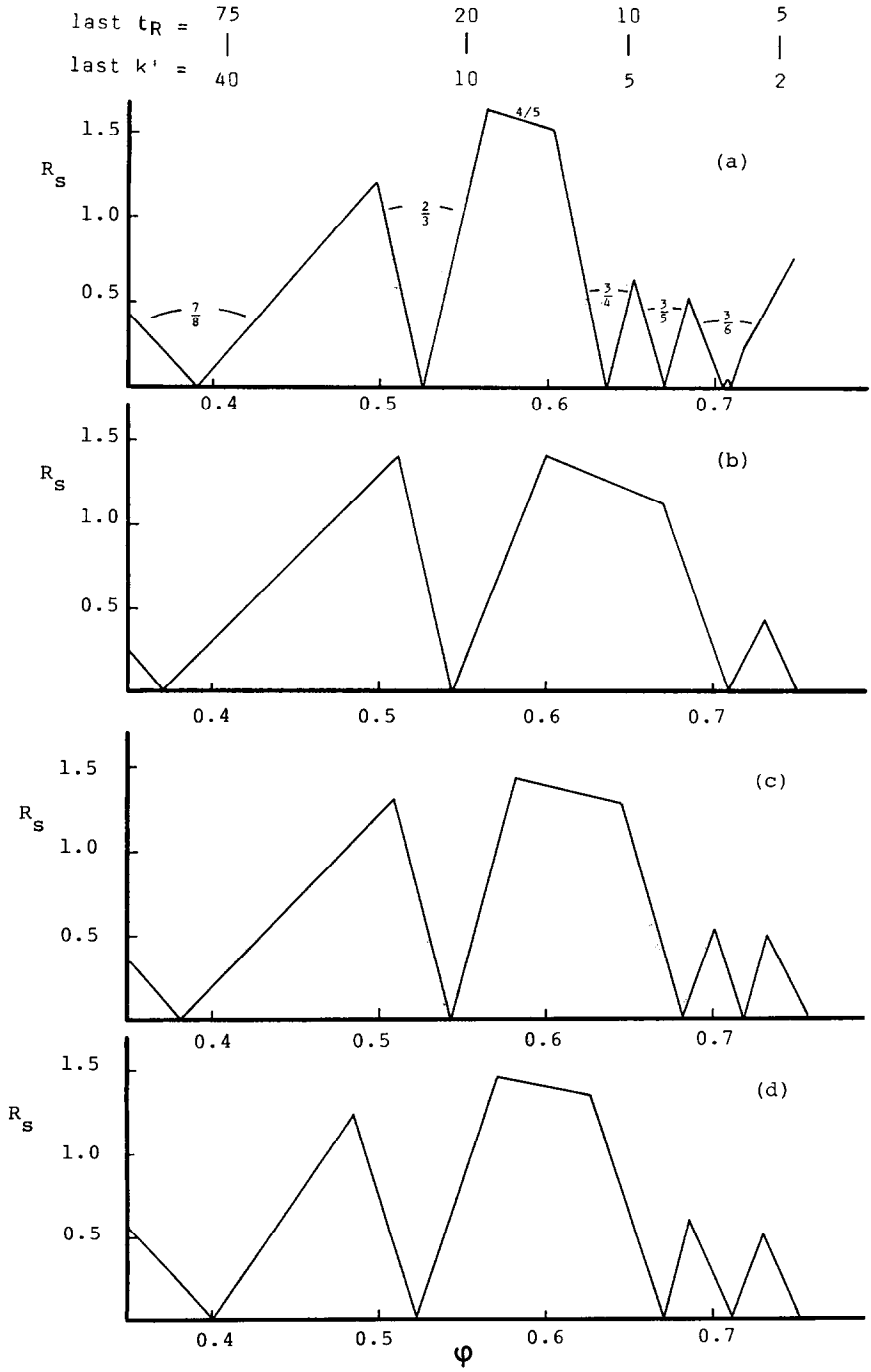
last t_R = 75 20 10 5
last k' = 40 10 5 2
R_S
1.5
1.0
0.5
(a)
(b)
(c)
(d)
7/8
2/3
4/5
3/4
3/5
3/6
0.4
0.5
0.6
0.7
φ

Fig. 4.

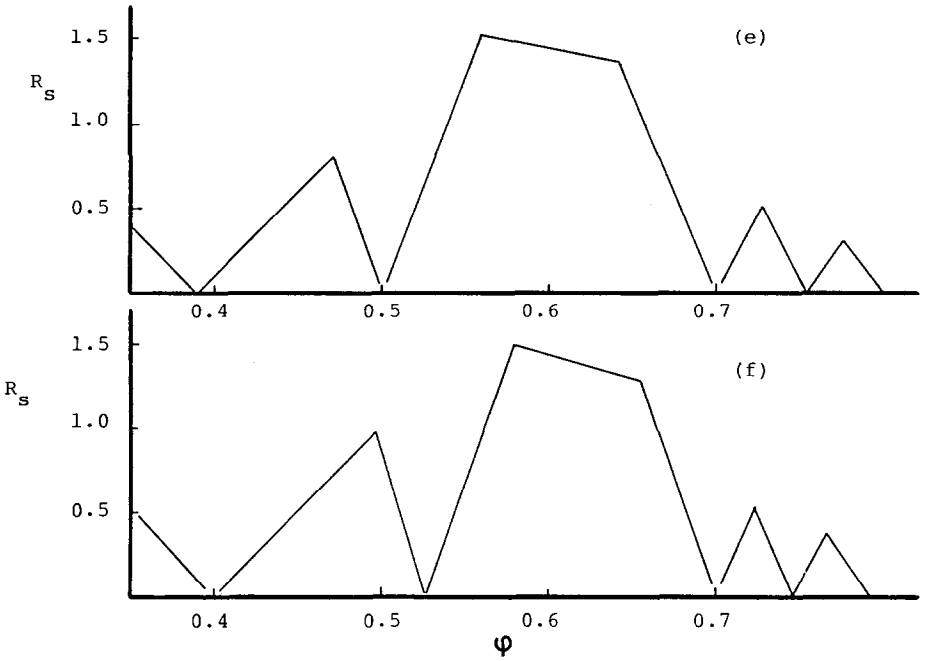

Fig. 4. Relative-resolution maps for the nitroaromatic sample in Table III and Fig. 1. Calculated for 10 000-plate column, using DryLab 45 software. (a) Derived from 20- and 40-min gradient runs; (b) 40- and 80-min gradient runs; (c) 20- and 80-min gradient runs; (d) isocratic runs with 40 and 60% methanol; (e) 50 and 70% methanol; (f) 40 and 70% methanol. t_R = Retention time in min.

these best values. Values of S are of primary interest, because they determine relative band-spacing as φ is varied. From Table IV we see that the errors in S are similar for both isocratic and gradient runs: 0.06–0.08 units for the gradient runs, and 0.04–0.06 units for the isocratic runs (*i.e.*, a 1–2% error in S). These errors should decrease when the two isocratic runs have more different values of φ, or when the ratio of t_G values for the gradient runs is larger[17], because in each case this corresponds to a larger change in k' between the two runs. When the average change in k' between the various pairs of runs (Table IV) is estimated, the error in S is seen to correlate well with this quantity. That is, the error in S is similar for both isocratic and gradient runs when the change in k' between the two runs is comparable. Also the error in S will decrease for larger ratios of the gradient elution time, or larger differences in percent organic solvent for two isocratic runs.

Errors in derived values of log k_w (Table IV) are also seen to be similar for either isocratic ($\pm$0.03–0.04) or gradient ($\pm$0.03–0.04) runs. Thus, when experimental conditions are comparable, gradient runs allow the prediction of isocratic data with an accuracy that is similar to that obtained by direct measurements of isocratic retention. That is, random experimental errors apparently have an equal effect on k' values measured either isocratically or by gradient elution[17,19,20].

Measuring resolution as a function of φ. The DryLab 45 program allows the user to determine relative resolution as a function of mobile phase composition (value

TABLE V

COMPARISON OF RESOLUTION-DIAGRAM PREDICTIONS BASED ON DIFFERENT GRADIENT AND ISOCRATIC RUNS

Application of DryLab 45 Program to data from Table III.

(a) Predictions of φ for $R_s = 0$

*Basis**	*Value of φ for overlap of indicated bands*					*Average error*
	7/8	*2/3*	*3/4*	*3/5*	*3/6*	
Best**	35%	53%	69%	72%	82%	–
20/40 min	37	54	71	75	81	2
40/80 min	39	53	64	67	71	4
20/80 min	38	54	68	72	77	2
40/60%	40	52	67	71	75***	3
50/70%	39	50	70	75	80	2
40/70%	40	53	70	75	79	

(b) Predictions of φ and R_s for maximum resolution

*Basis**	*Values of φ and R_s (in parentheses) for indicated bands*§					
	7/8, 2/3		*2/3, 4/5*		*3/4, 3/5*	
20/40 min	51%	(1.4)	60%	(1.4)	73%	(0.4)
40/80 min	50%	(1.2)	56%	(1.6)	65%	(0.6)
20/80 min	51%	(1.3)	58%	(1.4)	70%	(0.5)
40/60%	48%	(1.2)	57%	(1.4)	68%	(0.6)
50/70%	47%	(0.8)	56%	(1.5)	73%	(0.5)
40/70%	50%	(1.0)	58%	(1.5)	72%	(0.5)
Average	50%	(1.2)	58%	(1.5)	70%	(0.5)

* 20/40, 40/80 and 20/80 refer to different pairs of gradient runs (20/40 refers to calculations from 20-min and 40-min runs); 40/60% refers to isocratic runs with 40% and 60% methanol in water, etc.

** Calculations from best-fit lines to all isocratic data; see Table IV.

*** Isocratic predictions show bands 1/2 as the critical pair in this region of φ values (resolution for 1/2 and 3/6 are both small).

§ See Fig. 4a for R_s-maximum defined by 7/8, 2/3, etc.

of φ), beginning with data from either two gradient runs or two isocratic runs (Table III). Because only one separation variable (φ) is involved in resolution mapping, the results are conveniently represented by window diagrams[21]; *i.e.*, plots of minimum α *vs.* φ. Even more useful are corresponding relative-resolution maps (RRMs): plots of R_s for a 10 000-plate column *vs.* φ. Relative resolution is defined here as:

$$\text{relative } R_s = (1/4)\,(\alpha - 1)\,(10\,000)^{0.5}\,[k'/(1 + k')]$$
$$= 25\,(\alpha - 1)\,[k'/(1 + k')] \qquad (6)$$

The DryLab 45 program provides isocratic relative-resolution maps for each sample.

A RRM should be distinguished from a map of R_s *vs.* φ. The RRM is based on two experimental gradient (or isocratic) runs and assumes a column plate number of $N = 10\,000$ for all bands, whereas a plot of R_s *vs.* φ is based on data gathered from

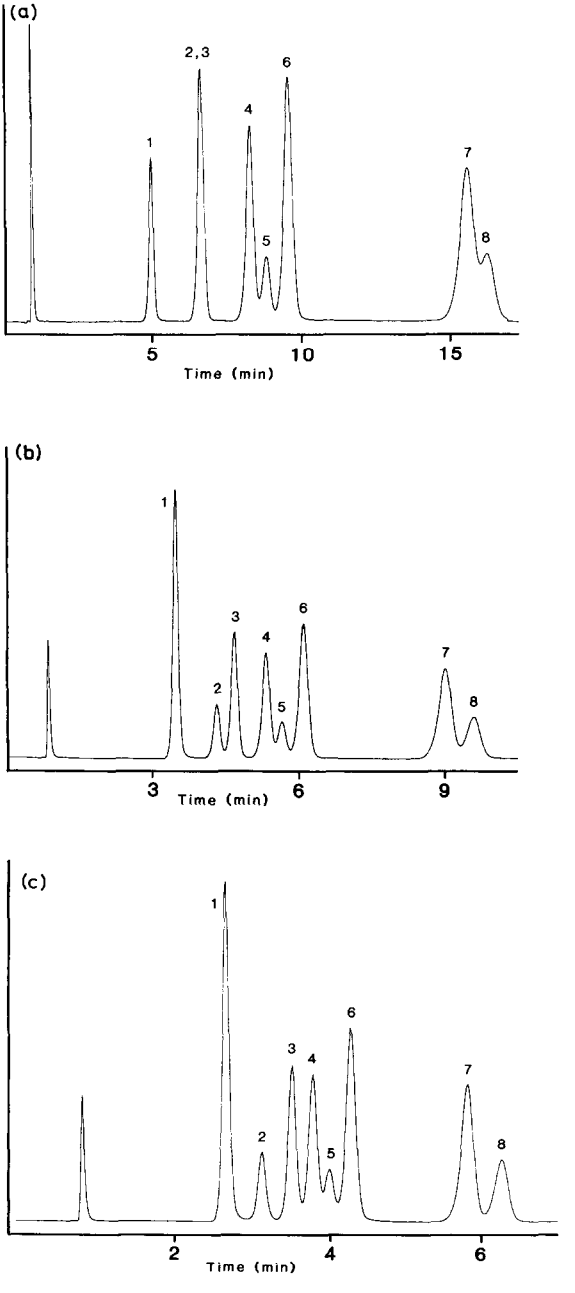

Fig. 5.

(Continued on p. 176)

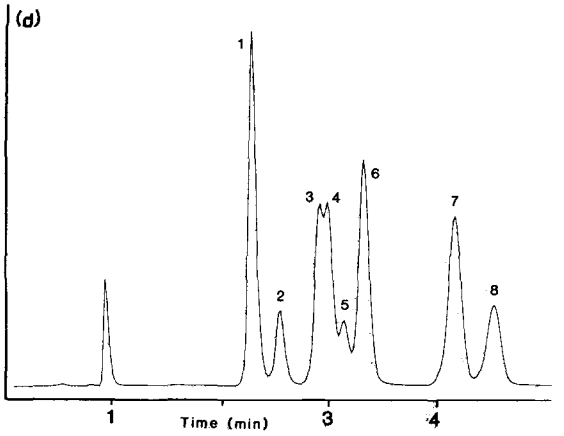

Fig. 5. Isocratic chromatograms of nitroaromatic mixture for different mobile phase compositions (values of φ). Column, two 8 × 0.4 cm, 5-μm Reliance C_8 cartridges in series; mobile phase, methanol–water; other conditions as in Table III. (a) methanol–water (54:46); (b) methanol–water (58:42); (c) methanol–water (61:39); (d) methanol–water (66:34).

a number of experimental isocratic runs in which N is measured for each band. The RRM is a very powerful tool, because it allows us to determine the resolution at one φ value *relative* to any other φ value, yet it only requires two experimental runs. Examples of RRMs for the present nitroaromatic sample are shown in Fig. 4, derived from various combinations of initial gradient or isocratic run pairs. It is seen that all of these plots are similar, reflecting the equivalent accuracy of DryLab 45 predictions, derived from either isocratic or gradient retention data. Table V presents further comparisons of the agreement between the various RRMs in Fig. 4. Mobile phases of minimum resolution (Table Va, unresolved bands) are predicted by the various RRMs with an average error of only ±3%. The accuracy of these predictions is somewhat better for the 20/80-min gradient runs, or isocratic runs with 40–70% methanol (because of a wider k' range). Likewise, predictions of mobile phases for maximum resolution are also in good agreement (Table Vb, ± 1 to 2% for the major resolution maxima).

Any of these RRMs from Fig. 4 allow the selection of an optimum mobile phase composition. Because we have seen that the data from runs with larger differences in t_G or φ are somewhat more accurate, we will use Fig. 4c (20/80-min gradient runs) as an example. A broad optimum in relative resolution is seen in the region of 58–64% methanol, with maximum resolution occurring for methanol–water (58:42). Because this optimum is rather flat, a good choice of φ would be about 60% methanol. This provides for reasonable resolution (R_s = 1.3–1.4), even if small errors (±2%) in the methanol composition should occur for any reason. Selection of the absolute R_s maximum at 58% would lead to variations in R_s from 0.6 to 1.4 for similar errors in φ. Fig. 5 shows isocratic separations at several values of φ; the separations for φ = 58% (Fig. 5b) and φ = 61% methanol (Fig. 5c) show acceptable resolution, as predicted by Fig. 4.

The DryLab 45 program also tracks analysis time or k' range for the sample as a function of φ (see Fig. 4a). These data can be considered together with RRMs

TABLE VI

GRADIENT RETENTION DATA FOR THE STEROID SAMPLE IN FIG. 3

Column, 25 × 0.46 cm, 6-μm Zorbax C_8; methanol–water mobile phase, 2 ml/min; 35°C.

Solute		*Gradient retention times (min)*			S^*		$\log k_w^*$	
		t_G = 10 min	t_G = 20 min	t_G = 40 min	20/40	10/20	20/40	
1	Prednisone	4.18	6.03	8.70	5.67	5.69	1.92	1.93
2	Cortisone	4.30	6.24	9.07	5.53	5.60	1.95	1.96
3	Hydrocortisol	4.45	6.52	9.61	5.47	5.62	2.00	2.04
4	Dexamethasone	5.05	7.67	11.80	5.33	5.52	2.25	2.31
5	Corticosterone	4.97	7.47	11.34	5.13	5.33	2.14	2.20
6	Cortixolone	5.17	7.85	12.07	5.07	5.30	2.21	2.29

* Values derived from two gradient runs of varying t_G; 10/20 refers to a 10-min and 20-min gradient.

in arriving at the final preferred separation conditions. When the mobile phase composition has been thus optimized by computer simulation (DryLab 45), column dimensions, flow-rate, and particle size[2] can also be optimized by computer simulation, using the DryLab program. This is further described in ref. 15.

Resolution of a steroid sample. The six-component sample of Fig. 3 was also tested by DryLab 45 computer simulation. The retention data from three initial gradient runs are summarized in Table VI, along with derived values of S and log k_w. Comparison of values of S and log k_w, derived from different pairs of gradient runs (20/40 or 40/80 min), shows agreement comparable to that seen in Table IV. That is, the data of Table VI provide a further illustration of the accuracy of DryLab 45 in predictions of isocratic separation *vs.* φ. Fig. 6a shows the corresponding RRM for this six-component steroid sample, calculated from both the 20/40 and 40/80 min gradient runs of Table VI. Predictions of resolution *vs.* φ are similar enough for selecting the optimum mobile phase composition: 32.5% organic solvent, with R_s

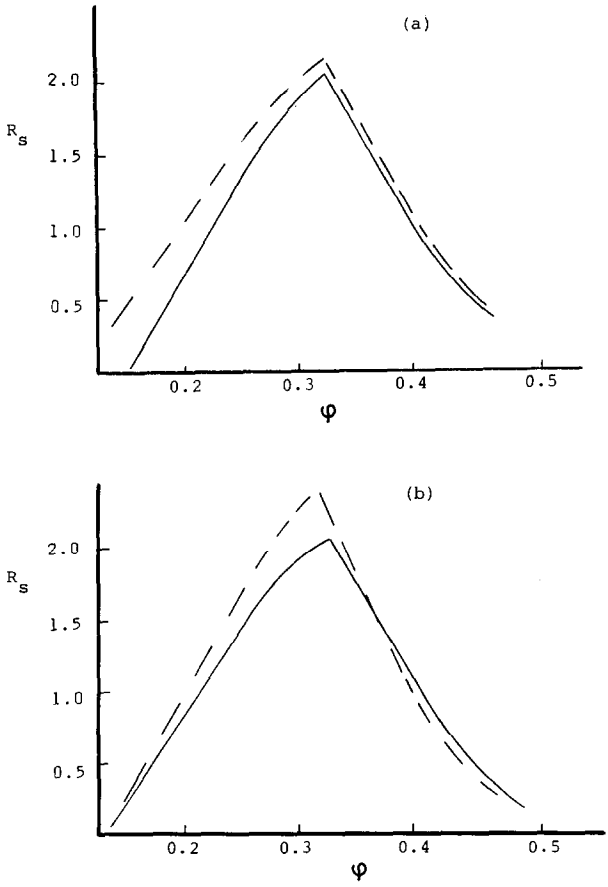

Fig. 6. Relative-resolution maps for steroid sample in Table VI and Fig. 3. (a) ———, 20/40-min runs; — — —, 40/80-min runs; (b) ———, 20/80-min runs; — — —, same as (a) except 10% error in V_D assumed (V_D = 6.25 ml; correct value, 5.75 ml).

= 2.0–2.1 for N = 10 000. Note that the use of other mobile phase compositions can result in significantly inferior separations in this case. Thus, even samples having components that are quite similar in molecular size and functionality can often benefit from φ optimization.

Maximizing the accuracy of gradient/isocratic predictions

Previous work summarized in ref. 17 has dealt with the steps that must be taken in order to obtain adequately reliable isocratic retention data from gradient runs. The present paper shows (with these precautions) that gradient-derived data can be as reliable as actual isocratic measurements. All the examples shown here are for small-molecule samples, with molecular weights less than 500. Larger molecules, such as proteins and synthetic polymers, require additional care for accurate gradient/isocratic conversions[17], but several studies have now shown that this procedure works equally well for high-molecular-weight compounds[17,22–25].

Measuring the dwell-volume of the HPLC system. Previously[22] we have suggested that the system dwell-volume V_D (volume from the gradient mixer to the column inlet) be measured from linear gradients, carried out without a column in the system. Some workers regard this as inconvenient. There is a possible alternative, which is facilitated by the DryLab 45 software. First, carry out two gradient runs, then use DryLab 45 to predict isocratic retention as a function of φ. Next an isocratic separation is performed that is predicted to yield convenient values of k' and analysis time. When the correct value of V_D is assumed, the isocratic retention data should agree best with the DryLab 45 predictions from the two gradient runs.

An error in V_D can result in significant errors in predicted isocratic retention. However, the effect on the resulting RRM is often less serious. This is illustrated in Fig. 6b, where the predictions based on the correct V_D value of 5.75 ml (solid line) are compared with predictions (broken line) based on a V_D value that is 10% larger. Work on the further analysis of possible errors related to imprecise V_D values and other sources is in progress.

CONCLUSIONS

The variation of solvent strength (percent water in the mobile phases for RP-HPLC) can lead to significant changes in band-spacing for many samples. This means that such samples can be adequately resolved by mapping resolution as a function of solvent strength. Method development based on this approach will generally be much faster than alternatives where different mobile phase solvents are investigated (solvent optimization), or where different HPLC columns are tried. This approach appears particularly promising in the case of samples having components that differ in molecular weight or functionality. However, even compounds of similar molecular size and chemical nature show useful changes in band-spacing as solvent strength is varied.

The use of solvent-strength optimization is facilitated by the use of initial gradient elution experiments at the beginning of method development. If two gradient runs are carried out in which only the gradient time is varied (*e.g.*, 20 and 60 min), the resulting retention data can be used to predict retention as a function of mobile phase composition (percent water) in corresponding isocratic separations (same sam-

ple and column). These predictions are most conveniently (and rapidly) carried out by computer simulations, using the DryLab 45 software package from LC Resources.

When the present approach is combined with computer simulation for optimizing column dimensions, particle size, and flow-rate (using DryLab 1–3 software), a very fast and powerful procedure for HPLC method development results. With only three or four actual laboratory experiments, plus perhaps half an hour of simulations using a personal computer, most samples should yield acceptable resolution for the purpose at hand. Method development carried out in this way is applicable to unknown samples, as well as samples with known components.

REFERENCES

1 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley-Interscience, New York, 2nd ed., 1979.
2 L. R. Snyder and P. E. Antle, *LC, Liq. Chromatogr. HPLC Mag.*, 3 (1985) 98.
3 S. R. Bakalyar, R. McIlwrick and E. Roggendorf, *J. Chromatogr.*, 142 (1977) 353.
4 N. Tanaka, H. Goodell and B. L. Karger, *J. Chromatogr.*, 158 (1978) 233.
5 P. J. Schoenmakers, H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, 185 (1979) 179.
6 J. L. Glajch, J. J. Kirkland, K. M. Squire and J. M. Minor, *J. Chromatogr.*, 199 (1980) 57.
7 P. J. Schoenmakers, H. A. H. Billiet and L. de Galan, *J. Chromatogr.*, 218 (1981) 261.
8 J. C. Berridge, *Techniques for the Automated Optimization of HPLC Separations*, Wiley-Interscience, New York, 1985.
9 P. E. Antle, A. P. Goldberg and L. R. Snyder, *J. Chromatogr.*, 321 (1985) 1.
10 P. E. Antle and L. R. Snyder, *LC, Liq. Chromatogr. HPLC Mag.*, 2 (1984) 840.
11 D. E. Henderson and D. J. O'Connor, *Adv. Chromatogr.*, 23 (1984) 65.
12 A. F. Poile and R. D. Conlon, presented at *8th International Symposium on Column Liquid Chromatography, New York, May 20–25, 1984.*
13 J. R. Gant, J. W. Dolan and L. R. Snyder, *J. Chromatogr.*, 185 (1979) 153.
14 W. R. Melander, B.-K. Chen and Cs. Horváth, *J. Chromatogr.*, 318 (1985) 1.
15 L. R. Snyder, J. W. Dolan and M. Rigney, *LC, Liq. Chromatogr. HPLC Mag.*, 4 (1986) 921.
16 J. L. Glajch, M. A. Quarry, J. F. Vasta and L. R. Snyder, *Anal. Chem.*, 58 (1986) 280.
17 M. A. Quarry, R. L. Grob and L. R. Snyder, *Anal. Chem.*, 58 (1986) 907.
18 X. Geng and F. E. Regnier, *J. Chromatogr.*, 332 (1985) 147.
19 M. A. Quarry, R. L. Grob and L. R. Snyder, *J. Chromatogr.*, 285 (1984) 1.
20 M. A. Quarry, R. L. Grob and L. R. Snyder, *J. Chromatogr.*, 285 (1984) 19.
21 R. J. Laub and J. H. Purnell, *J. Chromatogr.*, 112 (1975) 71.
22 M. A. Stadalius, H. S. Gold and L. R. Snyder, *J. Chromatogr.*, 296 (1984) 31.
23 E. S. Parente and D. B. Wetlaufer, *J. Chromatogr.*, 355 (1986) 29.
24 R. W. Stout, S. I. Sivakoff, R. D. Ricker and L. R. Snyder, *J. Chromatogr.*, 353 (1986) 439.
25 M. G. Kunitani, D. J. Johnson and L. R. Snyder, *J. Chromatogr.*, 371 (1986) 313.