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OPTIMIZATION OF MOBILE PHASES FOR MULTISOLVENT GRADIENT ELUTION LIQUID CHROMATOGRAPHY

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SUMMARY

Optimization procedures which have been previously developed for isocratic separations in liquid chromatography (LC) have been extended to include gradient elution systems. A multisolvent classification system is used which defines LC solvent systems based upon both solvent strength and selectivity considerations. A systematic experimental design is employed to gather basic retention data on the compounds in a mixture of interest. The data can then be fitted to a second-order polynominal surface and an overlapping resolution mapping technique is used to predict the optimum solvent system for selectivity purposes. Optimization of isoselective multisolvent gradient elution systems is the easiest and should be the most useful technique. A more powerful, but somewhat more complex, selective multisolvent gradient elution system is also described.

INTRODUCTION

Gradient elution liquid chromatography (LC) is a powerful method for separating complex mixtures of materials with widely varying retention characteristics. In traditional gradient elution LC separations, solvent strength is increased during the run, in a linear or non-linear fashion, so that all compounds in a mixture elute sharp peaks in a relatively short time. In a gradient elution separation optimized for solvent strength, all compounds in the mixture migrate with a capacity factor (k') of about 3 during the separation[1].

At present, gradient elution LC separations are utilized mainly in qualitative scouting studies or in situations where only a few samples are to be analyzed quantitatively. For routine quantitative analysis involving many samples, isocratic (constant strength) solvents are generally preferred over gradient elution. The latter technique does result in some decrease in precision because of problems in maintaining reproducible solvent strength during the gradient. Nevertheless, modern microprocessor-controlled instruments exhibit a good retention time precision ($< 1\,\%$ variation), so that relatively precise quantitative analyses now can be performed under gradient elution conditions when required.

We have recently discussed multisolvent LC mobile phase classifications that greatly expand the opportunity for optimizing solvent strength and selectivity effects

in LC[2]. Most of the gradient elution separations carried out to date are in a category that we have termed isoselective multisolvent gradient elution (IMGE). Here the ratio of the concentrations of organic modifiers to the carrier solvent (*e.g.*, water in a reversed-phase system) is increased during the separation, with the result that the solvent strength also increases. In terms of mobile phase selectivity, this system is analogous to typical isocratic LC separations; that is, the relative compositions of the organic modifiers to each other, and thus chemical selectivity, are not changed during the run. Consequently, the operator has to accept whatever selectivity is available with the particular organic modifier chosen.

One important aspect of gradient elution which has not been investigated so far, however, is the use of optimized mobile phases providing the widest possible selective interactions. Actually, in reversed- or normal bonded-phase LC, multisolvent mixtures of proton acceptor, proton donor, and dipole solvents may be required in the mobile phase carrier to provide the chemical environment for the best resolution of all peaks in a mixture[3]. Such an approach can produce the best compromise resolution of all the peaks in mixture with wide k' ranges. Although ternary gradients have been successfully applied, there has been no systematic study designed to optimize the selectivity of such solvent mixtures for gradients; separations largely involve laborious trial-and-error attempts to achieve a desired result. Since instrumentation is available for simultaneously mixing four solvents during a gradient separation[4], it is now practical to extend isocratic solvent optimization strategies previously used for the separation of substituted naphthalenes[3,5] and phenylthiohydantoin (PTH)-amino acids[6] to permit the total range of possible selectivity effects in gradient elution LC.

Finally, the most general type of solvent system, selective multisolvent gradient elution (SMGE), provides for changing both solvent strength and selectivity during the separation of mixtures containing compounds with a wide k' range. Solvent strength is increased while the selectivity of the mobile phase is varied, either continuously or in steps, to optimize the separation of various groups of compounds as the gradient elution chromatogram progresses.

In this study, we extend the optimization strategies previously used for isocratic separations in both bonded-phase and liquid–solid chromatography to include the important area of gradient elution separations. In this case, we use reversed-phase LC as a model system, but the approach is general for all LC systems. In contrast to traditional approaches, not only solvent strength, but also the selectivity of the mobile phase is varied to obtain adequate separation of all peaks in a mixture with a wide k' range in a reasonable time.

EXPERIMENTAL

Apparatus and reagents

All measurements were made with a Model 8800 liquid chromatograph (DuPont, Wilmington, DE, U.S.A.) which included a Model 870 three-headed pump with a four-solvent gradient mixer, a Model 850 fixed-wavelength UV photometric detector, a column compartment, and a Model 4100 recording integrator. Samples were injected with a Model 834 autoinjector which was programmed and controlled by the recording integrator. The 15 × 0.46 cm column (packed with

TABLE I

TEST COMPOUNDS

Code	*Name*	*Code*	*Name*
A	Resorcinol	H	Nitrobenzene
B	Theophylline	I	Cortisone
C	Phenol	J	Propyl paraben
D	Benzyl alcohol	K	Ramrod
E	Caffeine	L	Butyl paraben
F	Methyl paraben	M	Chloro-isopropyl N-(3-chlorophenyl)carbamate (CIPC)
G	Benzonitrile	N	Progesterone

Zorbax-C_8 chromatographic packing, DuPont) was operated at 35°C with a mobile phase flow-rate of 3.0 ml/min. The manufacturer's plate count specification of this column was confirmed in our laboratory.

Test solutes (Table I) were commercially available samples of compounds arbitrarily selected to produce a mixture having a k' range of about 100. Distilled-in-glass solvents (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) were used throughout the study. Retention time for components were determined by chromatographing individual components or simple mixtures of the complex mixture. Sample injection was initiated by programs available in the recording integrator. This device also actuated the microprocessor in the liquid chromatograph for carrying out the desired solvent mixing and linear gradient routines. Methods for the gradient runs stored in the LC microprocessor were initiated by the recording integrator at the desired time.

RESULTS AND DISCUSSION

Isoselective multisolvent gradient elution

IMGE involves changing the ratio of the carrier solvent (*e.g.*, water in reversed-phase systems) to organic modifiers during the separation so that the solvent strength changes during the run, although separation selectivity does not. A previously unexploited aspect of IMGE, however, is in the use of optimized multisolvent mobile phases to provide the best average resolution of all of the peaks in mixtures with wide k' ranges during a gradient run.

Optimized multisolvent gradients for mixtures with wide k' values may be determined using the same approach previously used for determining optimum solvent mixtures in isocratic separations[3]. The identical computer algorithm was utilized for determining the optimum multisolvent mixture. However, solvent strength was increased during the run, and retention times for the solutes were measured instead of k' values as in isocratic studies. The schematic in Fig. 1 represents the general solvent program approach used in the study. As in isocratic optimization, the reversed-phase solvent selectivity triangle uses methanol, acetonitrile, and tetrahydrofuran as the modifying solvents for different selectivity effects[3]. However, in IMGE, solvent strength is continuously increased during the run. Thus, the usual isocratic solvent selectivity triangle actually represents a constant solvent strength cross-section or slice of a solvent selectivity "prism" in which solvent strength is continuously increased.

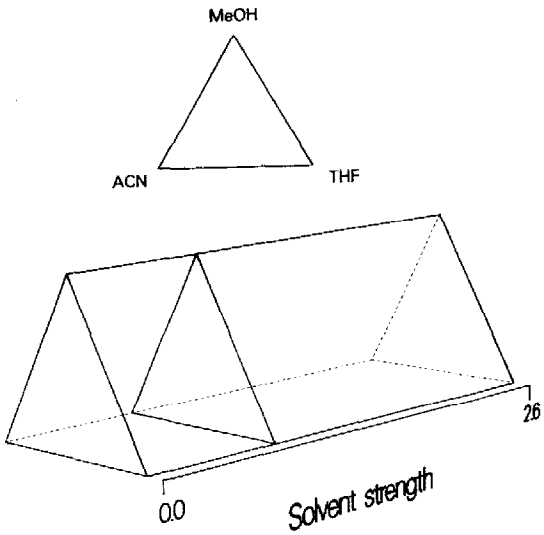

Fig. 1. Solvent strength prism for gradient elution compared with an isocratic solvent selectivity triangle for one solvent strength. ACN = Acetonitrile; MeOH = methanol; THF = tetrahydrofuran.

The slope of the solvent strength increase during the run can be varied. In reverse-phased gradient elution separations, solvent strength is normally increased linearly. In the case of Fig. 1, the solvent strength, S, values[2] of 0–2.6 arbitrarily represent a run in which the methanol modifier in water is varied from 0% to 100% by volume.

Accurate prediction of solvents for optimum selectivity in gradient elution is based on the systematic determination of the effects of the modifying solvents on relative retention times during the gradient increase of solvent strength. The experimental approach used to determine the effects of the various solvents on solute retention is very similar to that previously used for defining effects of solvent selectivity in an isocratic system[3]. Seven solvent mixtures were utilized in a form illustrated in Fig. 2 to acquire gradient elution retention data on all compounds in the mixture. First, binary solvent–water mixtures (mobile phases 1–3 of Table II) corresponding to each edge of the solvent selectivity prism were run with the test compounds using a

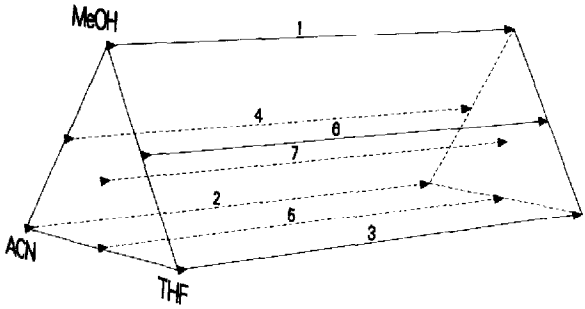

Fig. 2. Experimental design for seven gradient elution runs to obtain basic data for optimization calculation. Solvent compositions given in Table II.

TABLE II

MOBILE PHASE PROGRAMS

Mobile phase			*Solvent (volume %)*				
			Water	*Methanol*	*Acetophenone*	*Tetrahydrofuran*	
1		Initial	80	20	0	0	
		Final	0	100	0	0	
2		Initial	83	0	17	0	
		Final	16	0	84	0	
3		Initial	88	0	0	12	
		Final	41	0	0	59	
4		Initial	81	10	9	0	
		Final	8	50	42	0	
5		Initial	85	0	9	6	
		Final	28	0	42	30	
6		Initial	84	10	0	6	
		Final	20	50	0	30	
7		Initial	83	7	6	4	
		Final	19	33	28	20	
8 (Optimum IMGE)		Initial	83	2	14	1	
		Final	16	10	69	5	
9	*Step*						*Time (min)*
(Optimum step-selectivity)	1	Initial	84	10	0	6	2.5
		Final	76	15	0	9	
	2	Initial	82	0	0	18	4.5
		Final	72	0	0	28	
	3	Initial	55	24	21	0	13.0
		Final	8	50	42	0	

linear gradient of 20 min so that all compounds were eluted during the gradient, with the last compound eluting in about 15 min. Table II lists the initial and final volume percent of all mobile phase mixtures used during this optimization study. Next, runs were carried out with ternary solvent–water mixtures 4–6, representing equal volume mixtures of mobile phases 1 and 2, 2 and 3, and 1 and 3. Mobile phase 7 was composed of a one-third mixture each of solvents from the edge of the solvent selectivity–strength prism (1–3).

Data from gradient elution chromatograms with these seven mobile phase systems were used to estimate the coefficients of quadratic equations that describe resolution contour plots for each of the peak pairs in the mixture within the solvent selectivity prism. As with the optimization of isocratic mobile phases, this simple seven-mobile phase design produces data that satisfactorily define the resolution surface of the solvent strength–selectivity prism with an estimate of the experimental error for the lack of fit.

The results of all seven gradient elution mobile phase runs are listed in Table III and shown diagramatically in Fig. 3. The order of solvents in Fig. 3 has been selected to illustrate differences in selectivity for the various mobile phase solvents. As

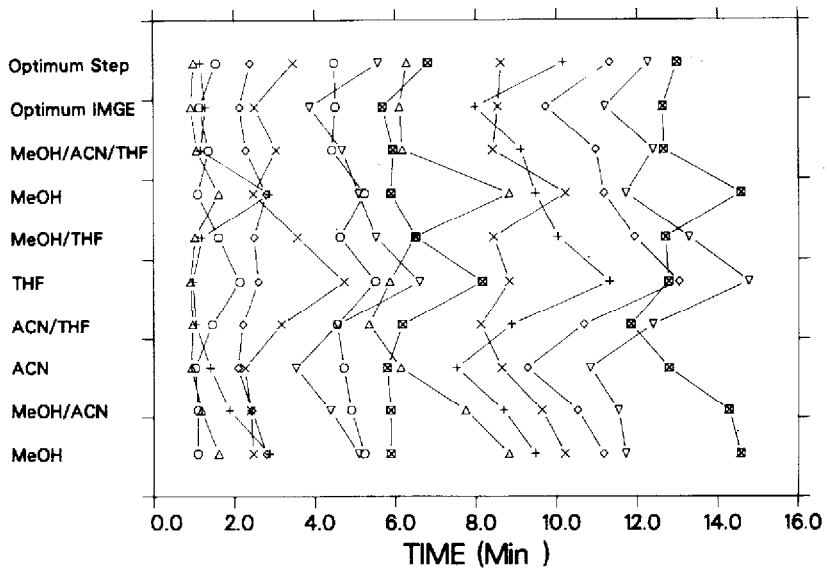

Fig. 3. Retention data for seven standard gradient elution runs and optimum IMGE and step-selectivity gradients. Retention data in Table II.

expected, significant differences in relative retention times are exhibited when the composition of the mobile phase is varied. Note that incomplete separation of at least one pair of compounds in the test mixture is observed for all seven mobile phases. This is illustrated in Fig. 4 for chromatograms obtained with the binary gradients in the seven mobile phase study. Similar results were obtained with the other four solvent runs; in no case were all the solutes completely separated, as tabulated in Table III and Fig. 3.

The overlapping resolution mapping (ORM) procedure of ref. 3 was used to identify the multicomponent solvent system producing the best compromise resolution of all the components in the test mixture. However, in contrast to isocratic separations where peak widths increase with retention time, peaks from a linear solvent strength gradient elution run have approximately the same width (standard deviation) throughout the run[7]. Therefore, in gradient elution, true plate count cannot be measured for a calculation of resolution of peak pairs in the mixture. In this study, *apparent* resolution based on the retention time differences of peaks, was utilized as a measure of separation quality in gradient runs.

The computer software for the ORM method used to determine the optimum gradient elution solvent system was the same as that in our study of optimized isocratic solvents[3]. The only change in the procedure involves using a constant increase in solvent strength, dS/dt, for the IMGE procedure, compared to the constant strength, S, required in isocratic separations. In other words, in IMGE the strength of the mobile phase is linearly increased (the slope of the gradient is held constant) compared to a single constant strength solvent used in the isocratic procedure. The routine in this computer program does not distinguish between retention times obtained on an isocratic or gradient elution basis. Consequently, selection of an optimum

TABLE III

COMPOUND RETENTION DATA

Compound	*Retention time (min)*									
	*Mobile phase**									
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*8P***
Resorcinol	1.10	1.04	2.14	1.10	1.46	1,61	1.37	1.14	1.55	1.10
Theophylline	1.62	0.94	0.92	1.19	0.97	1.03	1.07	0.93	1.00	0.99
Phenol	2.47	2.26	4.72	2.41	3.17	3.57	3.04	2.50	3.46	2.39
Caffeine	2.88	1.41	0.98	1.88	1.05	1.20	1.18	1.29	1.16	1.40
Benzyl alcohol	2.80	2.09	2.60	2.43	2.21	2.49	2.28	2.13	2.39	2.16
Methyl paraben	5.09	3.53	6.59	4.38	4.56	5.51	4.67	3.87	5.57	3.81
Benzonitrile	5.23	4.71	5.50	4.90	4.55	4.62	4.42	4.51	4.48	4.64
Nitrobenzene	5.89	5.79	8.15	5.88	6.17	6.50	5.94	5.68	6.81	5.77
Cortisone	8.82	6.13	5.86	7.74	5.34	6.48	6.17	6.10	6.29	6.36
Propyl paraben	9.47	7.52	11.31	8.68	8.88	10.03	9.11	7.98	10.15	7.94
Ramrod	10.21	8.63	8.82	9.63	8.11	8.43	8.41	8.54	8.62	8.74
Butyl paraben	11.17	9.27	13.03	10.52	10.67	11.92	10.97	9.73	11.32	9.74
CIPC	11.71	10.83	14.76	11.53	12.38	13.27	12.39	11.20	12.27	11.19
Progesterone	14.57	12.78	12.77	14.27	11.83	12.69	12.65	12.63	12.99	12.99

* As in Table II.

** Retention time predicted for mobile phase 8 by computer algorithm.

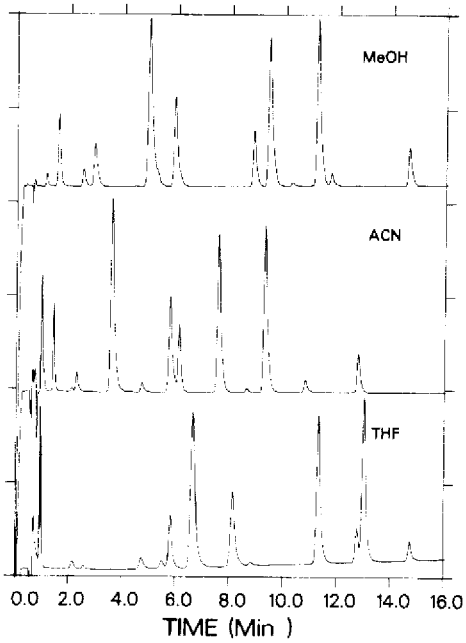

Fig. 4. Chromatograms of binary gradients (mobile phase systems 1, 2, and 3 in Table II).

IMGE solvent for the best compremise resolution of all components in a mixture is carried out in the same manner for gradient elution as for an isocratic separation.

The ORM technique relies upon measuring and comparing the resolution of every pair of peaks in the chromatogram obtained for each solvent from the seven-mobile phase program. From this, a resolution contour map is generated for each pair of compounds to estimate the apparent resolution of that pair in all solvent compositions within the selected solvent triangle. Thus, data from the seven solvent gradient runs actually permit the estimation of retention times for all peaks throughout the entire solvent selectivity prism when the gradient is maintained at a constant increase in solvent strength. The ORM method allows the cataloging of resolution for all pairs of peaks in the chromatogram by overlaying the apparent resolution for all pairs of compounds in the solvent triangle. The solvent system producing the largest minimum resolution of all peaks can then be predicted. Fig. 5 shows the three-dimensional overlapping resolution map obtained for the test mixture of Table I separated by IMGE. While other resolution peaks and valleys are noted in Fig. 5, the IMGE solvent producing the largest minimum resolution of all peak pairs in the mixture is clearly defined.

The IMGE solvent profile predicted as optimum in Fig. 5 is represented in the schematic of Fig. 6. As noted in Table II, initial solvent concentrations on the face of the initial solvent selectivity triangle for the optimized IMGE system were 83% water, 2% methanol, 14% acetonitrile, and 1% tetrahydrofuran. Solvent compositions at the end of the gradient were 16% water, 10% methanol, 69% acetonitrile, and 5% tetrahydrofuran. The solvent composition *versus* time plot for this IMGE separation is also shown in Fig. 6. In this case, the slopes of the composition plots for the modifying organic solvents (methanol, acetonitrile, and tetrahydrofuran) are maintained similar to provide approximately constant separation selectivity during the gradient elution run while solvent strength is linearly increased.

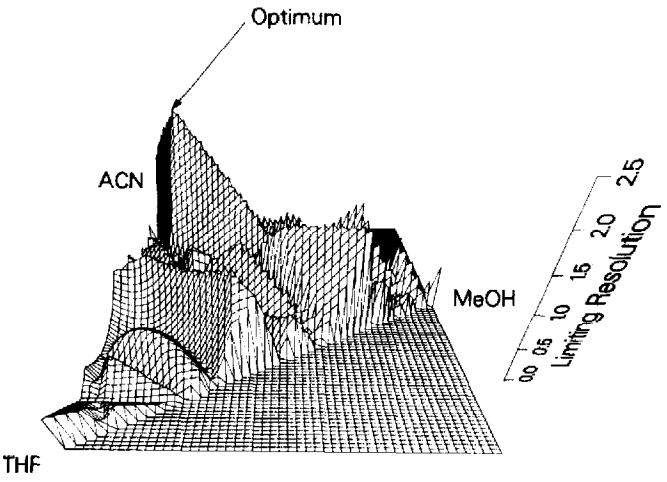

Fig. 5. Overlapping resolution map (ORM) for IMGE optimum. Optimum occurs at methanol–acetonitrile–tetrahydrofuran (12:80:8).

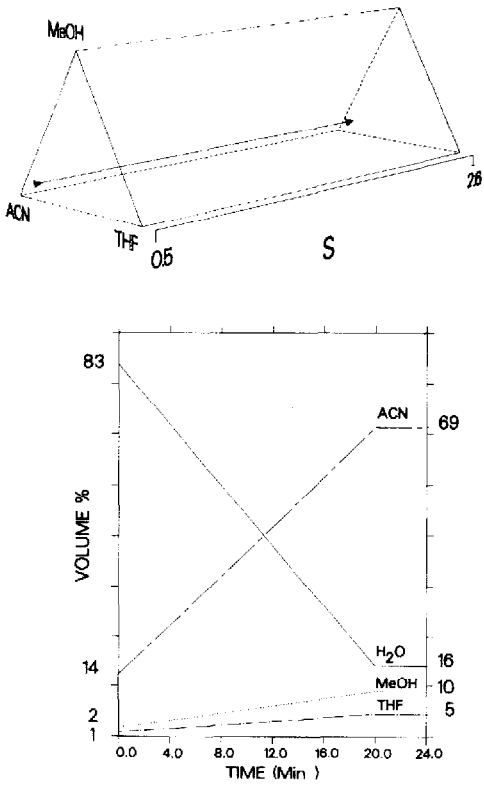

Fig. 6. Schematic representation of solvent program for optimum IMGE solvent system.

Fig. 7 shows the gradient elution chromatogram of the test mixture carried out with this optimum mobile phase system. Retention data are also given below in Table III and Fig. 3. Apparent resolution for each pair of peaks in the mixture was greater than 2.0, and the optimized separation required less than 15 min. This optimum IMGE chromatogram displayed significantly better resolution than any of the chromatograms obtained in the seven solvent runs, where at least one peak pair exhibited serious overlap in each case. The advantages of the IMGE separation for improved selectivity are clearly demonstrated for the quaternary solvent used to obtain this chromatogram, relative for those shown for conventional binary and ternary gradient (see Fig. 4). Several important crossovers in peak positions indicate significant potential for improved resolution and improved analysis accuracy.

It is possible that even higher resolution of the earlier peaks in the chromatogram of Fig. 7 could have been obtained by starting the gradient at a slightly lower S value. It is also likely that approximately equivalent resolution of the entire mixture could have been made with a reduced analysis time by operating the column at the optimum flow-rate (about 1 ml/min) using a gradient having a steeper solvent strength slope.

Retention times predicted by the statistical technique closely approximate experimental values, as indicated by the data for the optimum IMGE mobile phase system in column 8P in Table III. Uncertainties of 1–2% (one standard deviation) in

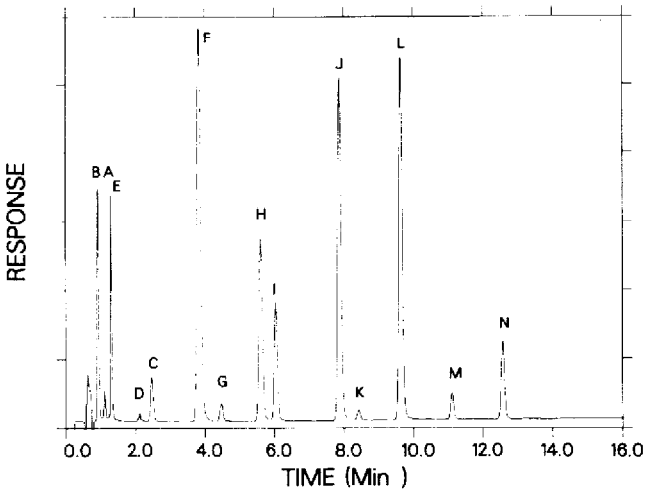

Fig. 7. Chromatogram for optimum IMGE run. Solute code in Table I.

retention times were typical in our studies, and this level of reproducibility demonstrates the ability of the technique to provide suitable data for quantitative analyses.

The simplicity of the IMGE approach makes it attractive for significantly improving resolution in gradient elution separations. Data required for estimating the optimum IMGE solvents can be acquired conveniently and rapidly. For example, all of the data required for the IMGE optimization herein discussed were obtained in a 26-h period of continuous and unattended instrument runs. Consequently, it was possible to run the optimum IMGE gradient in Fig. 7 shortly after the basic seven-mobile phase data were available. It appears from our work that the IMGE optimization involving quaternary solvents has the potential for substantial improvement in gradient elution separations with only modest additional effort compared with conventional approaches.

Selective multisolvent gradient elution

The most general type of solvent system in gradient elution is represented by SMGE, where solvent strength, selectivity, and composition are simultaneously varied during the chromatographic run[2]. Solvent selectivity changes during a gradient elution run are practical since later eluting peaks with large effective k' values remain near the inlet of the column and are essentially unaffected by solvent selectivity changes for earlier eluting peaks. Thus, SMGE is a powerful approach for optimizing the selectivity for several pairs or groups of compounds during a gradient elution separation of a mixture with a relatively wide k' range.

Advantageous solvents for a SMGE run can be conveniently determined by visual interpretation of data obtained in the seven-mobile phase statistical study. For example, inspection of the data in Fig. 3 suggested that a three-step change in selectivity with the usual linear increase in solvent strength would provide a superior separation of the test mixture. Fig. 8 shows the solvent selectivity prism for this SMGE step-selectivity gradient. Also given in Fig. 8 is the solvent concentration

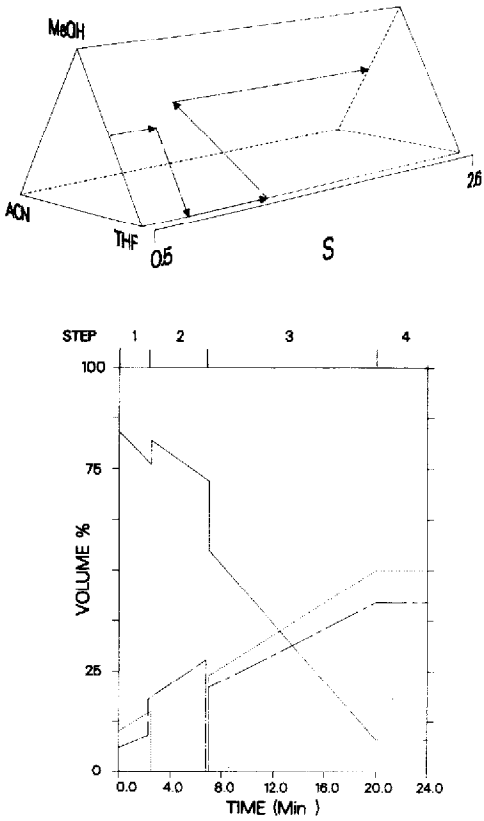

Fig. 8. Schematic representation of solvent program for step-selectivity gradient solvent system. ———, Water; · · · ·, methanol; – – –, acetonitrile; – · – · –, tetrahydrofuran.

versus time plot for this run. For the optimum separation, it was determined that the initial mobile phase should be the methanol–tetrahydrofuran–water ternary (mobile phase 6 of Table II). After 2.5 min, the solvent was changed to mobile phase 3, in this case, the tetrahydrofuran–water binary. This gradient was run for another 4.5 min, and the remaining compounds were eluted with the methanol–acetonitrile–water ternary (mobile phase 4) gradient.

Several interesting aspects can be noted for the chromatogram of the SMGE run shown in Fig. 9. First, all of the peaks are well separated, generally even better than in the IMGE run of Fig. 7. Second, substantial changes occur in elution order relative to the IMGE separation of Fig. 7. Such changes in elution order brought about by changing solvent selectivity during the gradient could be important in certain applications, although in this particular mixture there may not be any distinct advantage over the IMGE separation of Fig. 7. The advantage of changing selectivity during the chromatographic run for very difficult separations has already been widely exhibited in the ion exchange of amino acids[8], where changes in pH, temperature, and organic solvent composition have been used to produce improved separations for routine analyses.

A potential disadvantage of the SMGE approach is also illustrated in Fig. 9

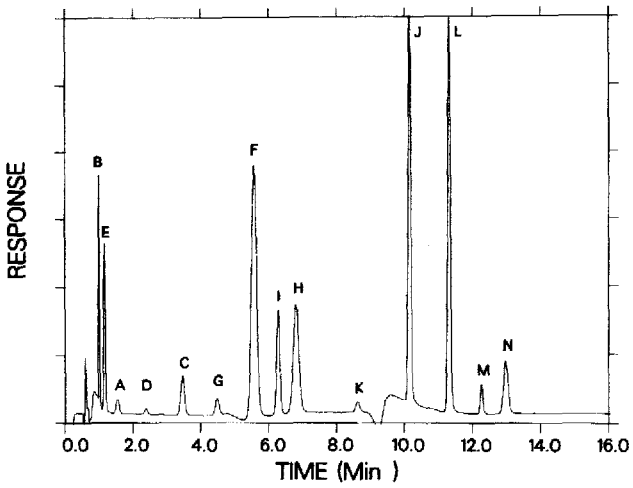

Fig. 9. Chromatogram for step-selectivity gradient shown in Fig. 8. Solute code in Table I.

with the baseline upsets occuring when the step changes in solvent selectivity were made, in this case, at about 4.8 and 9.8 min. Such upsets could be minimized by using highly purified solvents, but this would not eliminate the upsets caused by changes in mobile phase viscosity. However, as in this example, open spaces in the chromatogram can often be selected for such solvent change effects so that peak quantitation is not affected.

It should also be noted that the SMGE approach is not limited to step or abrupt changes in solvent selectivity. Linear or non-linear selectivity changes could also be incorporated simultaneously with linear or non-linear solvent strength increases to provide optimized separations. In this case, it might be predicted that detector baseline upset could be substantially reduced or eliminated by smooth rather than abrupt solvent changes.

The visual technique for "optimizing" solvents for the SMGE run of Fig. 9 could result in other solvent mixtures producing equivalent, perhaps even better, results, but with significant changes in peak elution patterns that might be beneficial for particular applications. The solvents arbitrarily selected for the Fig. 9 separation actually represent only one possible system. More precise estimations of optimum solvents can be accomplished by using a computer, and algorithms for such a system are currently under development.

We believe that the SMGE approach in which selectivity is varied during the gradient by changes in the mobile phase modifier can be a powerful tool for separating very difficult mixtures where analyses must be produced in large numbers and the development effort for an optimum separation is justified. Relative to the IMGE approach, SMGE is more involved and may be needed only in instances where the IMGE approach is not successful. In short, the IMGE approach using optimized solvents is easily structured to solve many separation problems involving mixtures with wide k' ranges. It is likely that the even more powerful SMGE approach will be needed only for the most difficult separations in which the additional sophistication can be justified.

Finally, it should be possible to predict the IMGE and SMGE solvents for optimum gradient runs in both normal bonded-phase and liquid–solid (adsorption) chromatography utilizing the approaches described above.

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REFERENCES

1 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley-Interscience, New York, 2nd ed., 1979, Ch. 16.
2 J. L. Glajch and J. J. Kirkland, *Anal. Chem.*, 54 (1982) 2593.
3 J. L. Glajch, J. J. Kirkland, K. M. Squire and J. M. Minor, *J. Chromatogr.*, 199 (1980) 57.
4 *TECHSCAN*, Vol. I, No. 3, DuPont Analytical Instruments Division, Wilmington, DE, 1981.
5 J. L. Glajch, J. J. Kirkland and L. R. Snyder, *J. Chromatogr.*, 238 (1982) 269.
6 J. L. Glajch, J. J. Kirkland and J. M. Minor, *32nd Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy*, Paper No. 330, 1981.
7 L. R. Snyder, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography, Advances and Perspectives*, Vol. I, Academic Press, New York, 1980, Ch. 4.
8 P. B. Hamilton, *Anal. Chem.*, 35 (1963) 2055.