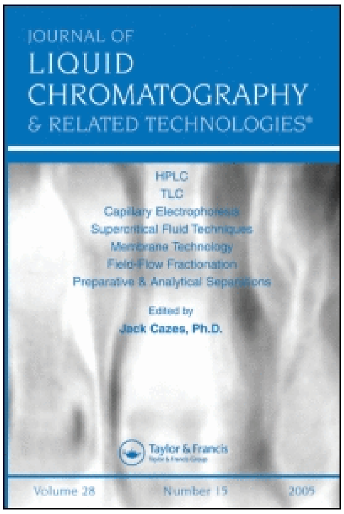

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A GRAPHIC REPRESENTATION OF BINARY MOBILE PHASE OPTIMIZATION IN REVERSED-PHASE HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A method for mobile phase selection for optimal separation in reversed-phase high pressure liquid chromatography is presented. The system is based on a plot of solute retention time versus binary mobile phase composition. A total of five data points are required. The method is simple and does not require a computer for data analysis.

INTRODUCTION

The selection of the mobile phase in high pressure liquid chromatography (HPLC) and thin layer chromatography (TLC) is by far the most critical step for a successful sample separation. The mobile phase not only determines the separation of the components in a mixture, but it also affects resolution and controls selectivity and the time of analysis.

Until recently the mobile phase was selected by trial and error based on the properties of the solute and the stationary phase. Systematic approaches

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to the selection of the mobile phase that will give optimum separation using normal and reversed phase TLC plates (1) and HPLC columns (1-4) have been published. The methods are based on statistical calculations, whereby peak pair resolution is plotted versus mobile phase composition. The resulting overlapping resolution mapping (ORM) plots indicate areas of maximum pair resolution. The union of the peak pair ORM plots will give the region where all resolutions are above a level predetermined by the analyst. Combinations of three pure, or mixed, solvents, and ten chromatographic runs with mobile phases of different solvent proportions are required to generate the experimental data base necessary for the subsequent statistical analysis.

The (ORM) approach works extremely well when three organic modifiers plus base solvent are necessary to achieve optimum resolution of all components of a complex mixture. It has been shown to be of wider application than the chromatographic optimization function (COF) method (2). The ORM approach can optimize resolution when only two organic modifiers and a base solvent are used. Belinky (5) used two organic modifiers and water to achieve separations in reversed phase HPLC. His system required 17 data points to achieve an optimum mobile phase. A simpler approach (6) with two organic modifiers was used which required only 10 data points. A computer program (1) is used to select the mobile phase which will give optimum resolution of the components in a mixture.

Recently (7), another approach to solvent optimization was published based on the linear relationship between log K' and log mole fraction of the solvent. This approach is not as sound or general as the statistical approaches discussed earlier.

In this study, a more practical approach to mobile phase optimization with two organic modifiers is presented. Only five chromatographic runs are required for the data base, and the subsequent mathematical treatment of the data is much less involved. The method is based on the window diagram technique which was originally developed by Laub and Purnell (8-11) for the optimization of separations in gas-liquid chromatography. Recently, a review of the window diagram application to GC, electrochemistry and spectroscopy was published (12). The technique has previously been used for the optimization of resolution of hydrophilic compounds with variation of the pH of the mobile phase in liquid chromatography (4,13). Contrary to the conclusion of Glajch et al (2) who dismissed the window diagram technique, the method is not limited to linear retention behavior nor to two-component solvent systems. Peak crossovers are also easily handled. In this work the method is successfully applied to the optimization of separation of a five-component mixture in reversed phase HPLC with two organic modifiers and water base solvents.

EXPERIMENTAL

Materials: Solvents were glass distilled (Burdick and Jackson). The chemicals were analytical grade (Aldrich Chemical Co.) and used without further purification.

Apparatus: A modular HPLC system consisting of Laboratory Data Control (LDC) constametric I and II Pumps attached to an LDS Gradient Master, a Chromatronix dual-channel uv absorbance detector, a Rheodyne injector, and a strip-chart recorder operated at 0.2 in/min was used.

The RP-18 reversed phase column was 250 mm x 4.6 mm prepacked with 10 μm particle size materials (Waters Associates). 10 μl samples were injected. Experiments were run at room temperature using a mobile phase flow rate of 1 ml/min. Retention times, peak widths (W) and resolutions (R_S) were determined with a 3352A Laboratory Data System (Hewlett-Packard) linked through a Hewlett-Packard 1865 A/D converter to the UV detector output of the liquid chromatograph. The output from the data system was recorded on a 9866A thermal line printer (Hewlett-Packard).

Separation Strategy:

The selection of the initial solvents (A and B) is based on the properties of the solute mixture and the stationary phase (normal, reversed phase or ion exchange). For reversed phase, the three most widely used solvents are acetonitrile/water, methanol/water and tetrahydrofuran/water. The initial ratios of organic/water selected are approximately 70-75% methanol/water, 60-65% acetonitrile/water and 40-50% tetrahydrofuran/water. The strategy for selecting two of these three is illustrated below. For simplicity, assume that a five component mixture is to be separated. The sample is first injected where 60% acetonitrile/water is the mobile phase. Should four peaks be obtained, standards are used to identify the two coeluting peaks. Only these two are then reinjected and eluted using a different solvent, for example, 50% tetrahydrofuran/water. Should two components be separated, then different mobile phase compositions are prepared using 60% acetonitrile as solvent A and 50% THF as solvent B. Should 50% THF/H_2O fail to separate the pair coeluting the percentage of THF is adjusted or another mobile phase is selected. This approach is simple and time saving because the analyst has only to separate the pair not resolved. Also, the identification of two components is simpler than identifying all components in a mixture.

This separation strategy was used to separate anthraquinone, 2-methylanthraquinone, 2-ethylanthraquinone, naphthalene and biphenyl. The sample solution was chromatographed with 60% AN/H_2O. Only three peaks were observed. Anthraquinone and naphthalene coeluted, as did 2-methylnaphthalene and biphenyl. However, both solute pairs were separated with 40% THF/H_2O. This demonstrates that each of the four pairs had been resolved in at least one of the initial solvents.

After selecting the initial solvents and the proportions of each in the three solvent combinations, the retention times data base is generated by recording the retention time of each solute in each of the different solvent combinations.

RESULTS AND DISCUSSION

The window diagram technique as presented by Laub and Purnell (8-11) is a graphical method for representing retention data. It was originally developed for the optimization of separations with respect to the binary stationary phase composition in gas-liquid chromatography. We have used the method to optimize separations in HPLC with respect to the mobile phase composition using two organic modifiers and a water-base solvent.

Table I shows the composition of solvents used and the retention times for each of the five solutes with each different mobile phase. The retention data

TABLE I: RETENTION TIMES FOR EACH OF THE SOLUTES AT EACH DIFFERENT MOBILE PHASE COMPOSITION

Mobile Phase Compostion						Retention Time, Min.				
			Actual Component Percentage							
Solvent	% A[a]	% B[b]	AN	THF	H_2O	Anthra-quinone	2-Methylanthra-quinone	Naphthalene	2-Ethylanthra-quinone	Biphenyl
1	100	0	60	0	40	7.23	8.98	7.23	10.94	8.98
2	75	25	45	10	45	8.26	10.30	9.16	13.27	11.77
3	50	50	30	20	50	9.57	11.98	11.30	15.82	15.13
4	25	75	15	30	55	11.65	14.75	14.67	19.98	20.38
5	0	100	0	40	60	16.44	21.20	22.41	30.19	34.03

(a) Represent 60% Acetonitrile (AN)/Water

(b) Represent 40% Tetrahydrofuran (THF)/Water

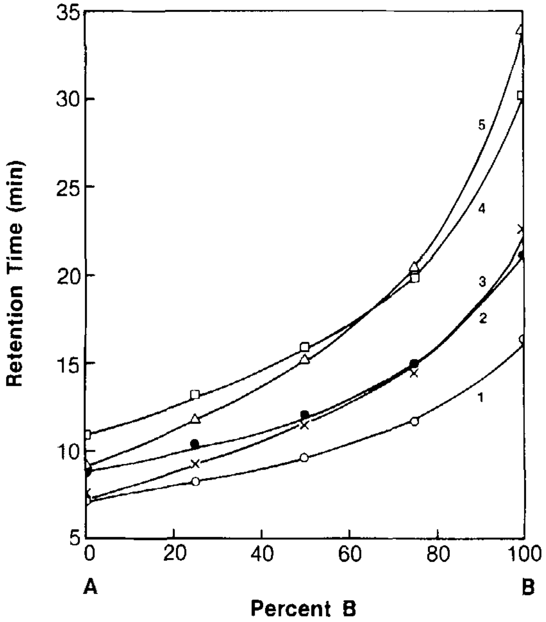

Figure 1: Retention time vs mobile phase composition for five solutes.

Solvents: A = 60% acetonitrile/water

B = 40% tetrahydrofuran/water

Solutes: 1 = anthraquinone;

2 = 2-methylanthraquinone;

3 = naphthalene;

4 = 2-ethylanthraquinone;

5 = biphenyl.

as a function of mobile phase composition was fit to a polynomial of the fourth order by least squares analysis.

Figure 1 shows plots of the calculated retention times for each solute as a function of mobile phase composition. The experimental points are also indicated. Note that, in contrast to gas-chromatography, the plots are not linear. This is due to the complicated nature of solute-mobile phase, solute-stationary phase and mobile phase-stationary phase interactions (14).

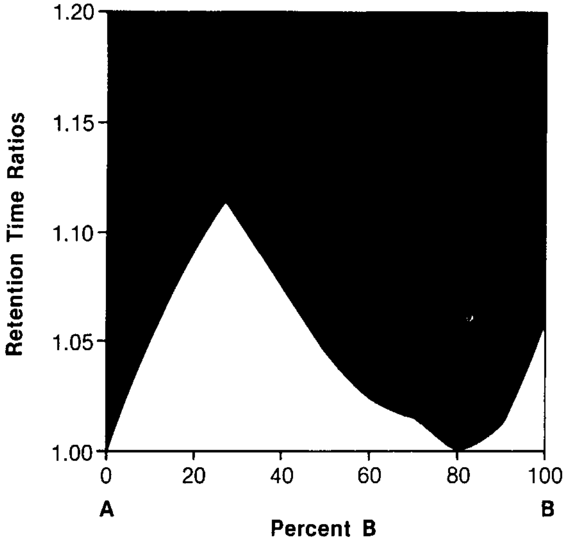

Figure 2: Window diagram for all ten pairs of five solutes, based on retention data as in Figure 1.

Figure 2 is a window diagram showing plots of retention time ratios versus mobile phase composition for all ten pairs of the five solutes. The region of retention time ratio values that are higher than the minimum found at each mobile phase composition is shaded. Note that when the relative retention is calculated to be less than unity (peak crossover) the reciprocal is taken such that the ratio is always greater than, or equal to, unity. The tops of the windows represent the mobile phase composition giving the best separation for the least separated pair. Two windows are seen in Figure 2, one at 27% B with a minimum retention ratio = 1.1, and a considerably smaller window (poorer separation) at 100% B. Thus the optimum mobile-phase composition for this particular separation is predicted to be 27% B (10.8% THF/43.8% AN/44.4%H_2O), which does, in practice, give base line separation of the components of the mixture (Figure 5).

The theoretical measure of separation of a solute pair in chromatographic techniques is the relative retention (α), which in HPLC is defined as the ratio

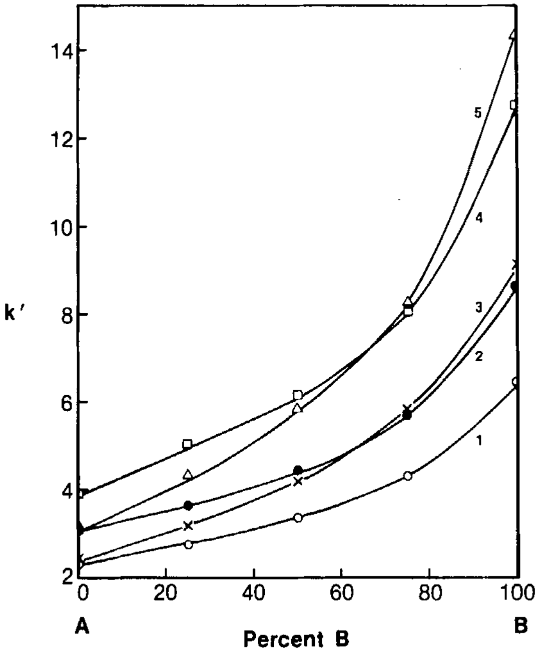

Figure 3: k' vs mobile phase composition for five solutes. Symbols for solvents and solutes as in Figure 1.

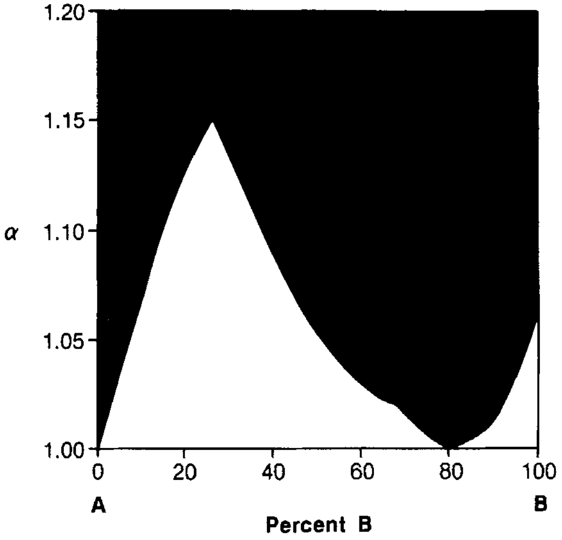

Figure 4: Window diagram for all ten pairs of five solutes, based on k' data as in Figure 3.

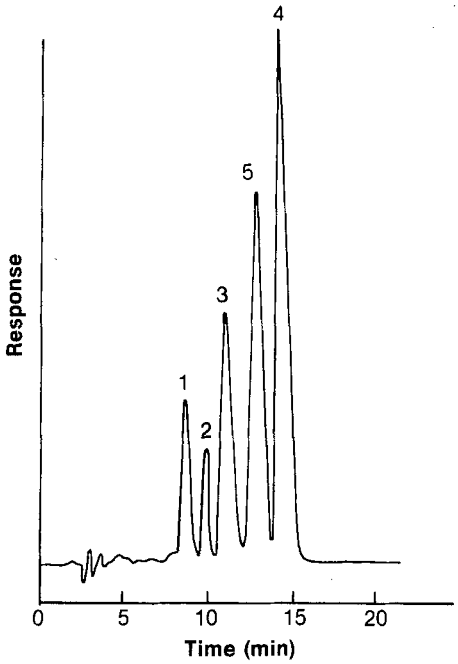

Figure 5: Chromatogram of the five solutes at optimum mobile phase composition (43.8% acetonitrile, 10.8% tetrahydrofuran, 44.4% H_2O) as determined from Figure 1.

of the capacity factor (k') of the more retained to the less retained solute. We calculated k' for all solutes at all solvent compositions by correcting for the column dead-volume. The k' data was treated in a similar way to the retention data of Table 1. Figure 3 shows plots of the calculated k' for each solute as a function of mobile phase composition, and Figure 4 gives the window diagram. Figures 1 and 3 and Figures 2 and 4 are strikingly similar. The optimum mobile-phase composition obtained from the larger window of Figure 4 is exactly the same as that obtained from Figure 2. However, here we can obtain the minimum value of α (1.15 at 27%B). Using this value we can calculate the minimum number of plates ($N_{req.}$) for separation according to Purnell's equation (15). In this instance, and assuming a capacity factor of five, $N_{req.}$ is calculated to be approximately 2350 plates. Note that accurate measurment of the column "dead volume" in HPLC is a difficult problem with no easy solution (16). Any optimization techniques dependent on k' data suffer from the unavailability of accurate methods for the determination of column dead volume. The window diagram method presented here does not require the accurate determination of k'. As demonstrated earlier the optimum solvent composition can be determined from raw retention time data. When retention time is plotted against mobile phase composition, it can be seen that a total of five runs (Figure 1) will give the mobile phase that will separate all the components of the mixture. It is clear that this approach requires no computer evaluation and is simpler than others (1-4) when two organic modifiers and a base solvent are used.

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