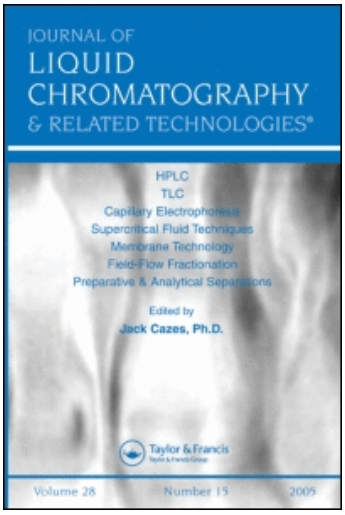

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Factorial Design in the Study of the Effects of Selected Liquid Chromatographic Conditions on Resolution and Capacity Factors

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FACTORIAL DESIGN IN THE STUDY OF THE EFFECTS OF SELECTED LIQUID CHROMATOGRAPHIC CONDITIONS ON RESOLUTION AND CAPACITY FACTORS

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ABSTRACT

Certain factors associated with the flow rate and minor components of the mobile phase are known to exert a significant effect on various liquid chromatographic parameters. By using the beta-phenylethylamine compounds ephedrine and (±)-norephedrine (phenylpropanolamine) as model solutes, the effects of pH, flow rate and concentrations of organic solvents and organic modifier in the mobile phase on solute peak resolutions and capacity factors were examined. A full factorial design was used and polynomial equations were derived to evaluate the quantitative relationships between the experimental conditions and the chromatographic parameters. A simple equation is proposed for the rapid estimation of resolution between solute peaks.

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INTRODUCTION

In high performance liquid chromatography (HPLC) optimization refers to the use of a statistically designed technique to minimize the number of experiments needed to establish the appropriate chromatographic conditions for adequate separation and acceptable elapsed time for sample elution. Snyder et al. (1) have discussed the theoretical basis for systemically optimizing and categorizing the strategies for retention optimization in HPLC and have classified them into one of three groups: (i) empirical (trial and error) approaches guided by experience and whatever theory is available, (ii) statistical design or computer search routines which allow intelligent guesses for successive changes in conditions, and (iii) development of an overall theory of retention as a function of the separation conditions that would permit the development of optimization schemes based on the preselection of well chosen independent parameters (chromatographic conditions), followed by interpolation of an optimum system for a given sample.

The independent variable most frequently employed in optimizing HPLC separations is the composition of the mobile phase (2). In the simplex design of Snyder and Glajch (2), an overlapping resolution mapping

technique is used to establish the optimum solvent mixture for the maximum resolution of all the adjacent bands in the chromatogram. On the other hand, De Smet et al. (3) have used a factorial design to study the effect of binary and ternary solvent systems having the same solvent strength but different selectivity on the resolution of peak pairs.

It is evident from the current literature that certain factors associated with components that are present in very small amounts in the mobile phase, such as the concentration of the organic modifier (4-9), or with pH and flow rate, exert a significant effect on the peak resolution (R) and capacity factor (k') of the eluents. Roos and Lau-Cam (4) have evaluated triethylamine (TEA) as a competing base for the resolution control and peak shape improvement in the reversed phase HPLC analysis of 174 drugs. Wahlund and Sokolowski (5) have studied reversed phase ion-pair liquid chromatographic systems for amines and quaternary ammonium compounds which include 1-pentanol as the organic liquid stationary phase, and aqueous solutions containing hydrogen phosphate, bromide or cyclohexyl sulfamate as counter ions. Good separations were obtained when N,N,N-trimethylnonylammonium or N,N-dimethyloctylamine were added to the mobile phase (5). In a separate paper (6), the same authors reported a

decrease in the retention time of several cyclic antidepressant amines when alkyl ammonium ions were added to a mobile phase that consisted of mixtures of methanol and buffers plus phosphate or bromide added as a counter-ion. Hung et al. (7) have investigated the reversed phase chromatographic retention of tricyclic antidepressant drugs with sodium laurylsulfate as the pairing-ion, both in the presence and in the absence of various organic amines, and have verified that the addition of an amine to the mobile phase will decrease the resolution of basic solutes and affect the selectivity of the stationary phase. Johansson et al. (8) described a procedure for the reversed phase paired-ion HPLC of organic ammonium compounds which used dihydrogen phosphate, bromide, cyclohexyl sulfamate or octyl sulfamate as counter-ions and 1-pentanol as the liquid stationary phase. These authors indicated that for the chromatographic separation of hydrophobic amines and of all types of quarternary ammonium compounds to occur, with negligible peak deformation, a long chain alkyl ammonium compound must be added to the mobile phase. Flangen and Jane (9) reported the factors that influence the retention, peak shape, and detector response of basic drugs during their HPLC analysis on silica columns.

The foregoing evidence clearly indicates that the resolution of peaks and retention values of the ana-

lytes are dependent not only on the major components of the mobile phase but also on the contribution made by other mobile phase-related factors such as the organic modifier, pH and flow rate. From a review of the literature it appears that such factors have received little attention in HPLC optimization.

Using ephedrine and norephedrine as the model drugs, the present work was undertaken to study the effect of selected liquid chromatographic variables on peak resolution and capacity factor by using a statistically designed approach.

EXPERIMENTAL

Apparatus

The chromatographic apparatus consisted of a constant flow reciprocating pump, a high pressure valve injector with a 20 uL sample loop, a Model UV 900 variable wavelength UV detector, and a Model LCI-100 integrator (Perkin-Elmer). Separations were performed on a 15 cm x 3.9 mm i.d. (Novapak C_{18}), column containing microparticulate-bonded (4 um) octadecylsilane material (Waters Associates).

Chemicals and reagents

HPLC grade acetonitrile and water (Fisher Scientific), sodium heptanesulfonate and triethylamine (TEA)

(Aldrich) ephedrine and norephedrine HCl (Fisher Scientific), and analytical reagent grade phosphoric acid and monobasic potassium dihydrogen phosphate (J.T. Baker).

Chromatographic conditions

1. Experimental design

To study the effect of the concentration of acetonitrile and TEA in, and the pH and flow rate of the mobile phase on the resolution and retention time of the eluents, a factorial design was performed using four variables at 2 or 3 levels of the following schedule: acetonitrile (10, 15 or 20%, v/v, 3 levels); TEA in aqueous phase (0.05 or 0.2%, v/v, 2 levels); pH (2.5 or 3.0, 2 levels); flow rate (1.5 or 2.5 ml/min, 2 levels) for a total of 24 experiments. Each experiment was performed in duplicate.

2. Mobile phases

The mobile phases were prepared by mixing appropriate volumes of acetonitrile and the aqueous phase so that the acetonitrile content was 10 to 20%, v/v. The aqueous phase was prepared by dissolving 0.005 M sodium heptane sulfonate and 0.02 M monobasic potassium phosphate in water containing 0.05 to 0.2%, v/v, of TEA. The pH of the mobile phase was adjusted to 2.5 or 3.0 by the addition of concentrated phosphoric acid.

RESULTS AND DISCUSSION

In a preliminary study it was determined that the detector response (as measured by the magnitude of the area or the height of the peak on the chromatogram) was maximum at the wavelength of 188 nm. Consequently all subsequent detections were carried out at this wavelength.

Ephedrine and norephedrine were selected as the model drugs because of their very close structural similarities and because of their widespread co-occurrence in many over-the-counter cold remedies.

Acetonitrile was used as the organic component of the mobile phase because of its low UV cutoff, low viscosity and higher boiling point than either methanol or ethanol. TEA was used to reduce peak tailing and shorten retention times. The pH of the mobile phase was adjusted to within a narrow range so as to evaluate the sensitivity of the method to pH changes. The flow rate was included in the design to verify if an increase in flow rate reduces the retention time of the solutes without significantly affecting peaks resolution.

Table 1 summarizes the experimental design and shows the levels of the independent variables (chromatographic parameters) studied along with the res-

TABLE 1

Experimental Values for Different Chromatographic Response Parameters at Various Experimental Conditions.

Expt. No.	Levels of independent variables				Measured responses					
					k_2'		$k_2' - k_1'$		R	
	CH_3CN[a] %	Flow rate, ml/min	pH	TEA[a] %	Mean	SD	Mean	SD	Mean	SD
1	10	1.5	2.5	0.05	21.34	0.15	6.31	0.03	4.40	0.04
2	10	2.5	2.5	0.05	24.69	0.06	7.34	0.01	4.41	0.04
3	10	1.5	3.0	0.05	25.60	0.04	7.60	0.02	5.19	0.18
4	10	2.5	3.0	0.05	25.87	0.17	7.68	0.02	4.74	0.22
5	10	1.5	2.5	0.20	22.69	0.12	6.34	0.07	5.54	0.07
6	10	2.5	2.5	0.20	21.95	0.36	6.07	0.07	4.86	0.18
7	10	1.5	3.0	0.20	19.11	0.20	5.35	0.05	4.37	0.03
8	10	2.5	3.0	0.20	20.61	0.05	5.78	0.02	4.41	0.22
9	15	1.5	2.5	0.05	7.12	0.15	1.95	0.02	3.65	0.02
10	15	2.5	2.5	0.05	7.14	0.01	1.97	0.01	3.23	0.26
11	15	1.5	3.0	0.05	6.93	0.09	1.90	0.03	3.52	0.20
12	15	2.5	3.0	0.05	7.68	0.04	2.12	0.01	3.23	0.40
13	15	1.5	2.5	0.20	5.77	0.03	1.55	0.01	3.46	0.31
14	15	2.5	2.5	0.20	6.09	0.08	1.63	0.02	3.25	0.16
15	15	1.5	3.0	0.20	5.88	0.15	1.49	0.14	3.84	0.28
16	15	2.5	3.0	0.20	5.92	0.02	1.58	0.01	2.96	0.28
17	20	1.5	2.5	0.05	2.54	0.01	0.63	0.01	2.34	0.01
18	20	2.5	2.5	0.05	2.59	0.01	0.64	0.01	1.62	0.10
19	20	1.5	3.0	0.05	2.63	0.01	0.66	0.01	2.15	0.01
20	20	2.5	3.0	0.05	2.69	0.05	0.66	0.01	1.72	0.15
21	20	1.5	2.5	0.20	2.15	0.01	0.50	0.01	1.75	0.13
22	20	2.5	2.5	0.20	2.16	0.04	0.49	0.01	1.42	0.16
23	20	1.5	3.0	0.20	2.13	0.01	0.48	0.01	1.81	0.28
24	20	2.5	3.0	0.20	2.18	0.01	0.50	0.01	1.67	0.47
Pooled SD						0.557		0.197		1.044
Pooled RSD of mean						0.114		0.040		0.213

[a] v/v basis.

ponses calculated from the corresponding chromatograms. The responses considered were the capacity factor of ephedrine (k'_2), the difference between the capacity factors of ephedrine (k'_2) and norephedrine (k'_1), and the resolution R. These parameters were calculated using equations 1 to 3.

$$k'_2 = (t_2-t_0)/t_0 \qquad \text{(Eqn. 1)}$$

$$k'_2 - k'_1 = [(t_2-t_0)/t_0] - [(t_1-t_0)/t_0] = (t_2-t_1)/t_0 \qquad \text{(Eqn. 2)}$$

$$R = 2(t_2-t_1)/(W_1+W_2) \qquad \text{(Eqn. 3)}$$

where: t is the retention time; k' is the capacity factor; W is the peak base width; t_0 is the retention time of the nonretained peak; and R is the resolution for two solute peaks. Subscripts 1 and 2 refer to the solutes with shorter (norephedrine) and longer (ephedrine) retention time, respectively.

The relationship between the particular chromatographic response (dependent variable) and the chromatographic parameters (independent variables) are shown by the polynomial equations 4 to 6.

$$R = 3.30 - 0.97\ m_1 - 0.18\ m_2 \qquad \text{Eqn. 4}$$

$$k'_2 = 10.56 - 6.88\ m_1 - 0.84\ m_3 \qquad \text{Eqn. 5}$$

$$(k'_2 - k'_1) = 2.38 - 1.99\ m_1 - 0.32\ m_3 \qquad \text{Eqn. 6}$$

where: m represents an independent variable related to the mobile phase, i.e., subscript 1 for the acetonitrile level, subscript 2 for flow rate, and subscript 3 for the TEA content in the aqueous phase. In the above equations the m values are the transformed values, which can range between -1 and +1. The relationship between the actual and the transformed value or experimental unit (eu) is expressed by equation 8.

$$1\ eu = [(\text{actual value}) - 1/2\ (\max + \min)] / [1/2(\max - \min)] \quad \text{Eqn. 8}$$

Equations 4 to 6 were derived following a typical method of construction of a polynomial equation using factorial design (10). The general form of the equation for the present four independent variables is:

$$\begin{aligned} Y = {} & B_0 + B_1n_1 + B_2n_2 + B_3n_3 + B_4n_4 \\ & + B_{12}n_1n_2 + B_{13}n_1n + B_{14}n_1n_4 + B_{23}n_2n_3 \\ & + B_{24}n_2n_4 + B_{34}n_3n_4 + B_{123}n_1n_2n_3 + B_{134}n_1n_3n_4 \\ & + B_{234}n_2n_3n_4 + B_{1234}n_1n_2n_3n_4 \end{aligned} \quad \text{Eqn. 7}$$

where: y is the chromatographic response of interest (dependent variable), Bs' are the coefficients and ns' are the chromatographic parameters (independent variables). The coefficients are related to the effects and/or interactions of the corresponding factors. A very small coefficient value indicates an insignificant

effect on a particular response and can be ignored. In equations 4 to 6 only significant terms are considered.

Equation 4 shows the resolution of two peaks as a function of acetonitrile content in the mobile phase and/or the flow rate. The negative value of a coefficient indicates that the response is inversely related to the factors, i.e., a decrease in the acetonitrile level and/or flow rate will increase the resolution of the peaks. Changing the pH of the mobile phase will have no effect on the peak resolution, as indicated by equation 4 which does not contain a pH term in it. The effects of the chromatographic conditions on k'_2 and $(k'_2 - k'_1)$ are expressed by equations 5 and 6, respectively. According to these equations both the k_2 and $(k'_2 - k'_1)$ values are only affected by the contents of acetonitrile and TEA in the mobile phase but not by a change in the pH or flow rate.

Figure 1 is a contour plot of resolution (R) and capacity factor (k'_2) as a function of the acetonitrile content and flow rate of the mobile phase. This figure clearly shows that both responses are very sensitive to the acetonitrile content in the mobile phase. The shaded area represents the region of interest and encompasses R = 3.0 to 3.5 and $k'_2 \leq 9$. These conditions should provide a good resolution in a reasonably short

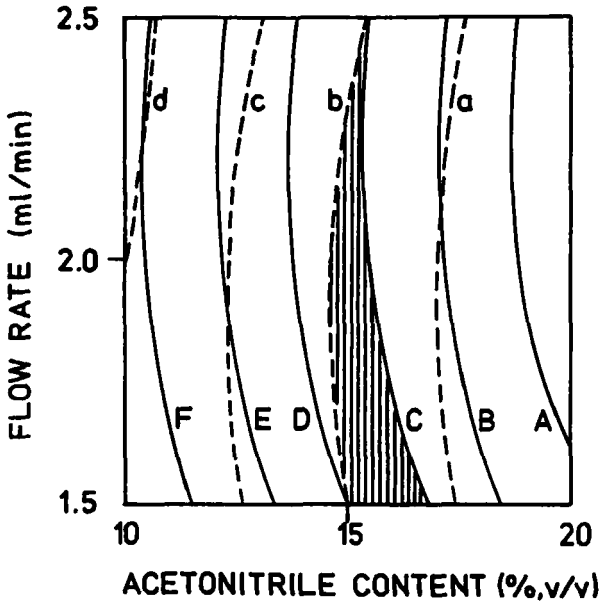

Figure 1. Contour plots for resolution (continuous lines) and capacity factor (broken lines) as a function of the flow rate of and the concentration of acetonitrile in the mobile phase. Key for resolution: A=2.0, B=2.5, C=3.0; D=3.5, E=4.0, F=4.5. Key for capacity factor: a=4, b=9, c=14, d=19.

run time. In addition, Figure 1 suggests the use of a lower flow rate when an increase in the flow rate will result in decreased resolution without a signi-ficant reduction in the value of k'_2, i.e., the run time.

Figure 2 shows typical chromatograms for minimum (A), optimum (B) and maximum (C) resolutions in minimum, optimum and maximum run times, respectively. It is evident from Table 1 and Figures 1 and 2 that a mobile phase containing 15% acetonitrile, 0.2% TEA and

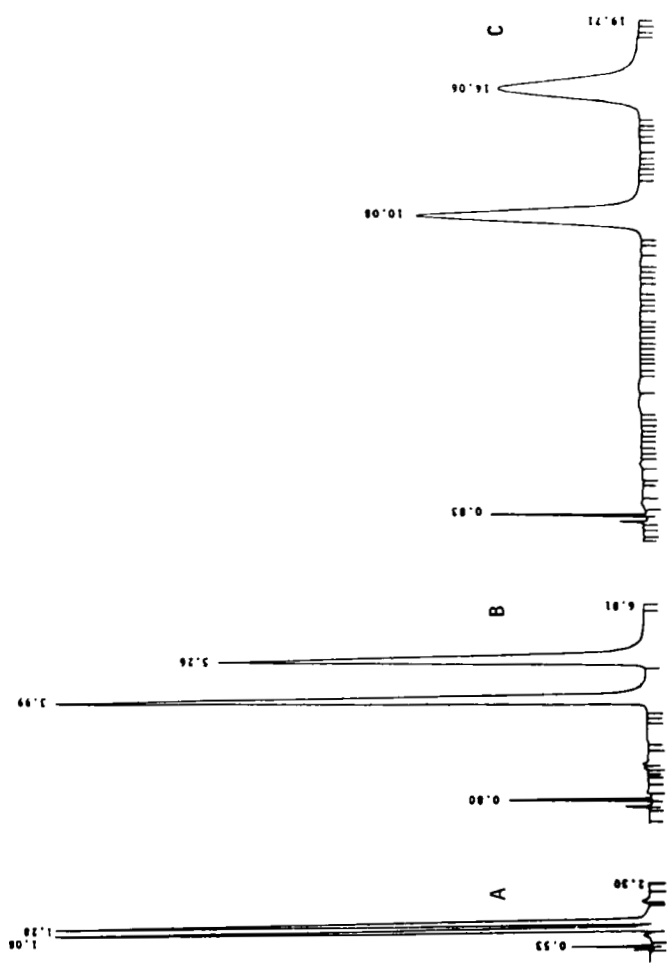

Figure 2. Chromatograms for different experimental conditions. Key: (A) acetonitrile 20% v/v, TEA 0.2% v/v, pH 2.5, and flow rate 2.5 ml/min.; (B) acetonitrile 15% v/v, TEA 0.2% v/v, pH 3.0 and flow rate 1.5 ml/min.; (C) acetonitrile 10% v/v, TEA 0.05% v/v, pH 3.0 and flow rate 1.5 ml/min.

TABLE 2

Correspondence Between Experimental and Predicted R Values

Expt. No.	Actual R value	Predicted R value	SE of Predicted	Lower 95 % of mean	Upper 95 % of mean
1	4.40	4.76	0.1138	4.52	5.00
2	4.41	4.94	0.1233	4.69	5.19
3	5.19	4.98	0.1244	4.72	5.24
4	4.74	4.99	0.1250	4.73	5.25
5	5.54	4.77	0.1140	4.53	5.00
6	4.86	4.71	0.1116	4.48	4.95
7	4.37	4.57	0.1049	4.35	4.78
8	4.41	4.65	0.1089	4.43	4.88
9	3.65	3.37	0.0725	3.22	3.52
10	3.23	3.38	0.0726	3.23	3.53
11	3.57	3.34	0.0725	3.19	3.49
12	3.23	3.47	0.0730	3.32	3.62
13	3.46	3.10	0.0736	2.95	3.25
14	3.25	3.16	0.0731	3.01	3.31
15	3.84	3.05	0.0742	2.90	3.21
16	2.96	3.12	0.0734	2.97	3.27
17	2.34	2.03	0.1063	1.81	2.25
18	1.62	2.05	0.1055	1.83	2.27
19	2.15	2.09	0.1039	1.87	2.30
20	1.72	2.09	0.1039	1.87	2.30
21	1.75	1.74	0.1202	1.49	1.99
22	1.42	1.74	0.1202	1.49	1.99
23	1.81	1.71	0.1214	1.46	1.96
24	1.67	1.76	0.1190	1.51	2.01

flowing at the rate of 1.5 ml/min will give $k'_2 \approx 7$ and $R > 3$ (see Experiment No. 15, in Table 1 and Figure 2B) which would be very satisfactory for the desired analysis. Since the chromatograms were unaffected by a change in pH from 2.5 to 3.0, the pH of the mobile

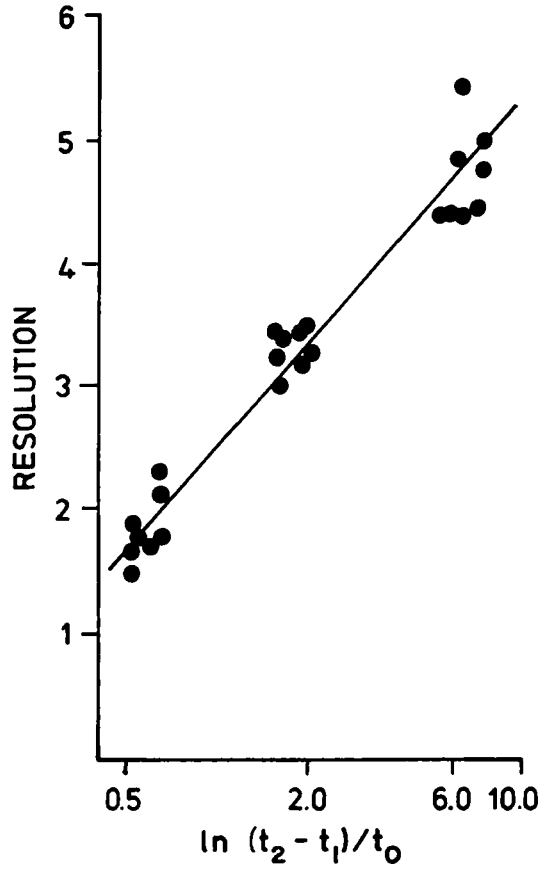

Figure 3. Plot of resolution versus ln $(t_2 - t_1)/t_0$ for 24 chromatographic runs under various experimental conditions.

phase should be kept preferably at 3.0 to minimize its detrimental effect on column selectivity.

Prediction of resolution

Currently available equations for calculating R values are complicated by the fact that they make use

of either the number of theoretical plates or the width of the peak bases, parameters which are not easily measurable. In an attempt to find a simpler means of estimating resolution, Eqn. 8 was found to provide good linearity as shown in Figure 3.

$$R = 1.18 \ln [(t_2-t_1)/t_0] + 2.58 \qquad \text{Eqn. 8}$$

where: 1.18 and 2.58 are the slope and intercept values of a plot of R versus $\ln (t_2-t_1)/t_0$, respectively, $(t_2-t_1)/t_0 = (k'_2 - k'_1)$ (see equation 2). Table 2 shows the good agreement between the actual and calculated values of R and the 95% confidence limits, a finding which supports the usefulness of the equation. However, predetermination of the slope and intercept value from a plot of R versus $\ln [(t_2-t_1)/t_0]$ will be required for particular combinations of solute and mobile phase.

CONCLUSION

The statistically designed experimental approach presented here examined the quantitative effect of various chromatographic parameters on the peak resolution and capacity factor of the solutes. For the range of pH values and flow rates of the mobile phase studied, it appeared that these parameters exerted at negligible effect on the responses. This information

could be of help to the analyst who is trying to establish the most appropriate chromatographic conditions during the development of an HPLC method. The proposed equation will be useful in estimating the resolution of peaks from the retention times of the solutes and the nonretained peak.

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