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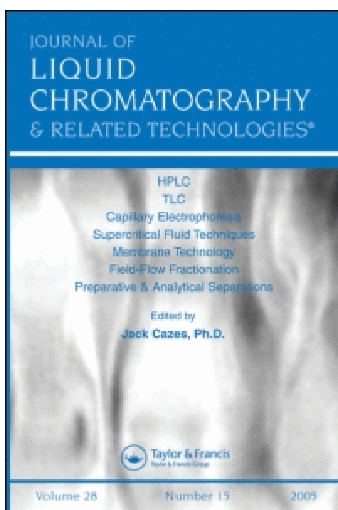

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Evaluating HPLC Assay Robustness with Experimental Design

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ABSTRACT

A twelve run screening factorial experimental design was used to study the instrumental robustness of an HPLC weight percent assay for a fermentation derived pneumocandin B_0. The factors varied were the instrumental settings of wavelength, injection volume, flow rate, mobile phase composition, column temperature, and column lot. The measured responses were the fundamental liquid chromatographic parameters of: retention time (RT); capacity factor (k'); theoretical plates (N); tailing factor (T); and resolution (Rs). The effect of each factor on the responses was calculated and significance determined by analysis of variance (ANOVA).

Key Words: HPLC assay robustness; Plackett–Burman design; Statistical evaluation.

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INTRODUCTION

Robustness of an HPLC procedure is the effect of small, deliberate changes in method parameters, and is determined subsequent to the optimization of an assay.[1] Information concerning assay robustness is useful for troubleshooting problems as they arise, particularly when transferring analytical methods between instruments or laboratories. Knowledge of the factors that a particular assay is sensitive to can be of tremendous value when correcting a test method that is not performing as expected. For this reason, instrumental robustness as outlined by the International Conference on Harmonization (ICH),[1] is investigated in our laboratories as part of the validation process before transferring HPLC methods to the manufacturing setting. In this case, the instrumental settings of column temperature, flow rate, wavelength, injection volume, mobile phase composition, and the manufacturer column lot are investigated for their effect on column efficiency parameters of retention time (RT), theoretical plates (N), tailing factor (T), capacity factor (k'), and resolution (Rs). Column efficiency or fundamental LC parameters, are often used as system suitability requirements.[2] System suitability requirements[3] are established to determine whether an assay as a whole (equipment, operations, and samples) is performing as expected, and can be expressed by a particular measurement or measurements.

Factorial experimental design is a powerful method of obtaining information from a limited number of experiments. Many recent applications of factorial experimental design to chromatography including optimization,[4–7] mathematical modeling,[8–10] mechanistic studies,[11–14] and robustness[15–27] are found in the literature. Screening designs[28] are a common factorial experimental design used for determining the robustness of a process. In this paper, the procedure for using a twelve-run screening design to determine the effects of small changes to instrumental settings on column efficiency parameters, and applying analysis of variance to assess their significance, are outlined. For a more detailed overview of screening designs as a robustness test, see Ref.[15].

The twelve-run screen is a Plackett–Burman design[29] using a two-level, nongeometric, orthogonal array, and is applied to determine the ruggedness of laboratory test methods.[30] The array is shown on the left side of Table 1. Each column of the table contains six −s and six +s corresponding to the factor levels, which are values slightly lower and higher than the instrumental settings specified by the previously developed and optimized assay. The rows of the table are the experimental pattern that contains the instrumental settings for the twelve chromatographic runs performed. The values of the factor levels are given in Table 2.

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Table 1. Experimental design array, responses, and effects.

	Factors						Responses				
Run	Temperature	Flow	λ	Organic percent	Injection volume	Column	RT	N	T	k'	Rs
1	+	+	−	+	+	+	13.576	5,877	1.00	24.33	1.46
2	+	−	+	+	+	−	13.796	5,964	1.15	23.64	1.34
3	−	+	+	+	−	−	12.991	5,532	1.14	23.51	1.39
4	+	+	+	−	−	−	22.223	6,574	1.16	40.62	1.77
5	+	+	−	−	−	+	22.654	7,266	1.02	41.50	1.78
6	+	−	−	−	+	−	23.578	6,852	1.15	40.88	1.66
7	−	−	−	+	−	+	14.111	6,135	1.01	23.93	1.46
8	−	−	+	−	+	+	23.748	7,071	1.00	40.66	1.75
9	−	+	−	+	+	−	13.008	5,462	1.12	23.27	1.34
10	+	−	+	+	−	+	14.320	6,502	1.03	24.30	1.53
11	−	+	+	−	+	+	22.376	6,674	1.00	40.51	1.69
12	−	−	−	−	−	−	23.110	6,499	1.14	40.27	1.71
			Effects								
RT	0.13	−0.97	−0.10	−9.31	0.11	0.35					
N	277	−273	38	−911	−101	440					
T	0.02	−0.01	0.01	−0.00	−0.01	−0.13					
k'	0.52	0.01	−0.16	−16.91	−0.14	0.51					
Rs	0.03	−0.00	0.01	−0.31	−0.07	0.08					

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Table 2. Factor levels.

	−	0	+
Temperature	28°C	30°C	32°C
Flow rate	1.65 mL/min	1.70 mL/min	1.75 mL/min
Wavelength	209 nm	210 nm	210 nm
Organic percent	35	36	37
Injection volume	9 μL	10 μL	11 μL
Column lot	T71071		T71331

The assay under investigation is an isocratic weight percent determination of pneumocandin B_0. Pneumocandin B_0 is a macrocyclic compound whose structure[31,32] is given in Fig. 1. The conditions of the assay are: Waters NovaPak C18, 150 × 3.9 mm thermostated at 30°C; water : acetonitrile, 64 : 36 (v/v), at 1.7 mL/min; UV detection at 210 nm; and an injection volume of 10 μL. The critical separation for this assay is between pneumocandin B_0 and the serine analog of pneumocandin B_0.[33] The resolution between these two peaks is the measured response for Rs. A chromatogram of the separation of pneumocandin B_0 and the serine analog run under the conditions of the assay is shown in Fig. 2.

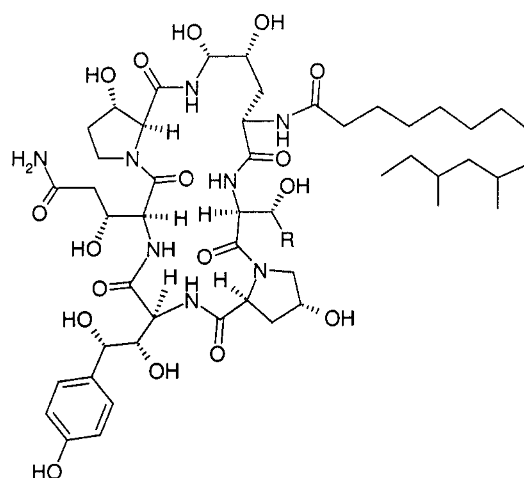

Figure 1. Structures of pneumocandin B_0 and its serine analog.

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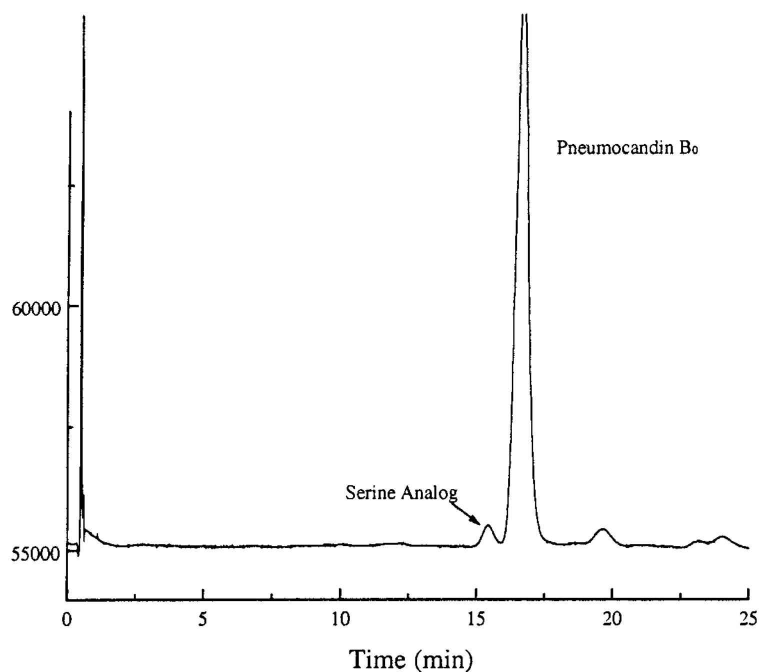

Figure 2. Typical chromatogram.

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EXPERIMENTAL

Instrumentation

The HPLC runs were performed using a Hewlett Packard HP1050 Series Chromatograph (Avondale, PA). The operating parameters for each run are given by the pattern in Table 1 using the values in Table 2. The columns employed were Waters Novapak C18, 150 × 3.9 mm ID, 4 μm (Phase Separation Corporation, Franklin, MA). For the purpose of this work, the first peak in the chromatogram was assumed to be the void time (t_0). Data acquisition and analyses, including k', N, T, and Rs were performed using PE Nelson ACCESS*CHROM 1.9.5 software (Perkin-Elmer Nelson Systems, Inc., Cupertino, CA) operating on a MicroVAX 3100/20e, computer (Digital Equipment Corporation, Norwalk, CT). Analysis of variance (ANOVA) and effects calculations were performed with programs written in Microsoft EXCEL.

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Reagents

HPLC grade solvents were obtained from Fisher Scientific (Springfield, NJ), and the water used was purified with a Hydro Water Management System (Garfield, NJ). A sample of pneumocandin B_0 containing 2.6% (area) of the serine analog was supplied by the Bioprocess Research and Development Department of Merck Research Laboratories (Rahway, NJ).

RESULTS AND DISCUSSION

For each of the twelve runs, the responses for RT, k', N, and T for the pneumocandin B_0 peak were determined, as well as Rs between the pneumocandin B_0 and the serine analog. These data are found on the right side of Table 1. Table 1 also contains rows at the bottom referred to as effects. The effects[34] are calculated by subtracting the mean of the responses obtained from the low factor level from the mean of the responses obtained from the upper factor level. Thus, the effect, or data contrast, of the factor "temperature" on the response "k'" is calculated as follows:

$$\text{Effect} = \frac{24.33 + 23.64 + 40.62 + 41.50 + 40.88 + 24.30}{6} - \frac{23.51 + 23.93 + 40.66 + 23.27 + 40.51 + 40.27}{6}$$

$$\text{Effect} = 32.55 - 32.03 = 0.52$$

Temperature was determined to have a positive effect 0.52 on k'. The physical significance of this is that one would expect that an increase of temperature from the method's listed temperature of 30°C, would result in an increase in k', and a decrease in k' would be observed if the temperature was slightly lower than 30°C.

The positive effect of temperature on k' is an unexpected result. The opposite effect is typically observed with reverse phase chromatography, and k' decreases as temperature increases. In a separate study, this effect was confirmed by determining the k' under the conditions of the assay, while varying the temperature from 28°C to 32°C. A slight positive effect of temperature, within the range studied, was confirmed as shown in Fig. 3.

The experimental design allows us to obtain statistical information about the calculated effects for the fixed factors of temperature, flow rate, wavelength, percent organic, and injection volume. Analysis of variance were performed[35,36] to determine whether or not the calculated effects for these factors are statistically significant. The same statistical analysis cannot be applied to the choice of

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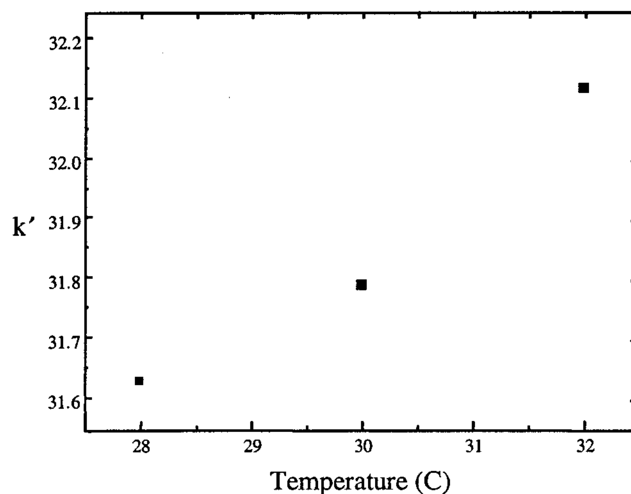

Figure 3. Temperature vs. k'.

column as this represents two lots of many production lots. The columns were varied during the experiment design, thus allowing some inferences to be made. Table 3 contains the calculated ANOVA tables.

The data were evaluated at 2 significance levels, 5% and 1%. The F value would have to exceed 5.99, and 13.75, respectively, for the factor to be considered significant. As an example, the F value for the effect of temperature on k' is 5.52; thus, it is not significant at the levels tested.

The effects found significant at the 5% level are significant at the 1% level. From the data in Table 4, temperature, wavelength, and injection volume have no effect on the measured responses. It is clear from the instrument parameter that most effects the chromatography of the pneumocandins is the percent organic. The effect of this factor on retention time, theoretical plates, capacity factor, and resolution is significant down to the 1% level. In addition, the effect of flow is predictably significant on retention time.

The positive effect of temperature on k' is not significant up to the 5% level. If the ANOVA results were evaluated at higher significance levels, the effect of temperature on k' would be the next effect deemed to be significant. However, any result at a higher level is less meaningful. While this effect may be observed, the magnitude of the shift, ~0.5, from 28°C up to 30°C is relatively small compared to the calculated capacity factors (~32). Such a small shift would indicate that the method is relatively rugged with respect to small changes in temperature, and in fact, the chromatography is acceptable over the range studied.

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Table 3. ANOVA tables, critical values of F: 5.99 (5% SL); 13.75 (1% SL).

Source	df	SS	MS	F
		Capacity factor (k')		
Temperature	1	0.81	0.81	5.52
Flow rate	1	0.00	0.00	0.00
Wavelength	1	0.07	0.07	0.50
Percent organic	1	857.84	857.84	5,843.18
Injection volume	1	0.06	0.06	0.40
Error	6	0.88	0.15	
Total	11	859.67		
		Tailing factor (T)		
Temperature	1	0.00	0.00	0.09
Flow rate	1	0.00	0.00	0.02
Wavelength	1	0.00	0.00	0.02
Percent organic	1	0.00	0.00	0.00
Injection volume	1	0.00	0.00	0.06
Error	6	0.05	0.01	
Total	11	0.05		
		Theoretical plates (N)		
Temperature	1	230,797	230,797	1.95
Flow rate	1	223,423	223,423	1.90
Wavelength	1	4,264	4,264	0.04
Percent organic	1	2,488,488	2,488,488	21.10
Injection volume	1	30,846	30,846	0.26
Error	6	708,876	118,146	
Total	11	3,685,430		
		Resolution (Rs)		
Temperature	1	0.00	0.00	0.86
Flow rate	1	0.00	0.00	0.01
Wavelength	1	0.00	0.00	0.08
Percent organic	1	0.28	0.28	72.55
Injection volume	1	0.01	0.01	3.43
Error	6	0.02	0.00	
Total	11	0.32		
		Retention time (RT)		
Temperature	1	0.05	0.05	0.56
Flow rate	1	2.84	2.84	29.43
Wavelength	1	0.03	0.03	0.29
Percent organic	1	260.28	260.28	2,700.11
Injection volume	1	0.04	0.04	0.39
Error	6	0.58	0.10	
Total	11	263.81		

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Table 4. Significant effects.

	Temperature	Flow	Wavelength	Percent organic	Injection volume
RT	—	*	—	*	—
N	—	—	—	—	—
T	—	—	—	*	—
k'	—	—	—	*	—
Rs	—	—	—	*	—

*Significant at both 5% and 1% levels.

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While the ANOVA cannot be used for determining the significance of the effect of the column on the measured responses, inferences can be made by analyzing the calculated effects. By inspection, the two production lots of this column differ in efficiency, as seen by the effects the columns on theoretical plates (*N*) and tailing (*T*); see Table 1.

For the purposes of this assay, all twelve runs gave suitable separations of the two compounds of interest, indicating that this method is fairly rugged to small instrumental changes. The ranges of measured effects can be used to set typical values of column efficiency parameters that may be applied as system suitability requirements. Based on the responses found in Table 1, one might set system suitability parameters of: RT > 13 min; $N > 5500$; $T < 1.2$; $k' > 23$; and Rs > 1.3.

Based on the significance of each factor found in Table 4, instrument settings may be modified to correct a failed system suitability parameter. For example, the number of theoretical plates may be increased with a slight decrease in the organic content of the mobile phase. This local optimization is acceptable, provided that all other system suitability parameters are met. However, an effective way of decreasing the peak tailing can be made by a change in columns. This parameter is probably useful in determining a column's effectiveness over time.

CONCLUSION

A screening experiment provides an efficient method for evaluating instrumental robustness by determining the factors that most effect chromatography and their statistical significance. With that knowledge, the proper controls can be placed on the least robust factors. In addition, information gained from the experiment can be used to assign system suitability requirements for a particular test method.

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