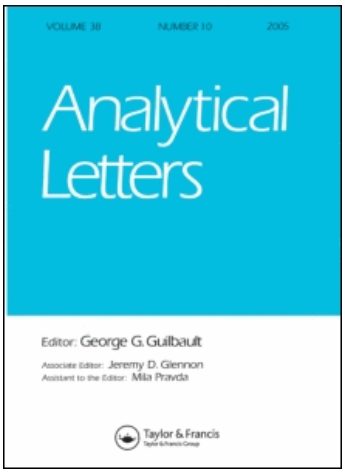

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The Application of a Three Factor Central Composite Experimental Design to The Paper Chromatographic Separation of Several Amino Acids

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THE APPLICATION OF A THREE FACTOR CENTRAL COMPOSITE EXPERIMENTAL DESIGN TO THE PAPER CHROMATOGRAPHIC SEPARATION OF SEVERAL AMINO ACIDS

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KEY WORDS: Central Composite Design, Paper Chromatography, Amino Acids, Experimental Design

ABSTRACT

A three factor central composite design was used to study the effect of methanol, propanol and amyl alcohol on the paper chromatographic separation of arginine, histidine, leucine, threonine and tryptophan. Statistical analysis of the R_f data yields second order equations which describe the response surface of R_f as a function of the composition of the 3 alcohols. This data is useful in understanding the relative influence of each alcohol and in predicting R_f values for different solvent combinations.

INTRODUCTION

Paper chromatography was one of the earliest techniques used for separations of amino acids[1,2,3]. This is because of the fact that amino acids, along with flavonoids are the two classes of compounds most amenable to separation by paper chromatography. This is due to their favorable solubility characteristics in a

wide range of convenient developing solvents, and the availability of suitable papers and sensitive detection agents such as ninhydrin.

Smith[1] lists a table of about 400-500 amino acids, indoles, imidazoles and guanidines in several solvents including a butanol, acetic acid, water mixture (120:30:50). He also reviews the literature and shows several 2 phase, 2 way chromatographic systems to further resolve complex amino acid mixtures. Hundreds of solvents for amino acids and their derivatives have been tabulated[4] and reviewed[5]. Recently a 2^5 factorial design[6] was used to study the effect of n-C_1 to C_5 alcohols on the R_f of these same amino acids.

Two goals of response surface methodology are (1) to find an approximate function (of low order polynomials, usually second order) to predict future responses and (2) to determine factor responses which optimize the response function. A central composite design is usually used because (1) all coefficients of a second order model can be estimated, (2) lack of fit can be analyzed and (3) criteria such as orthogonality and rotatability can be satisfied. Box and Wilson[7] were the first to develop central composite designs in their studies of chemical processes at ICI. Response surface methodology[8-11] has been developed and expanded since then and is a primary tool for the scientist wishing to understand his system better. An added benefit comes when the understanding of the optimum region (where factor levels produce the maximum or minimum response) yields relevant tradeoffs as to cost or performance and where knowledge of the response surface shape can be used to direct experiments in a region which would not be explored otherwise.

EXPERIMENTAL

The paper chromatography was done on Whatman #1 chromatography paper. The alcohols were Analytical grade and were

obtained from Mallinckrodt. Arginine, Leucine and Threonine were obtained from Matheson, Coleman and Bell, Histidine from Sigma Chemical Company and Tryptophan from United States Biochemical Corporation. Ninhydrin for visualizing the spots was obtained from Baker. A solvent of 50 to 75% alcohol with the remaining percentage being 1:1 glacial acetic acid/water was previously studied. This suggested the levels of -1 being 15% and +1 being 25%. The alcohols used were C-1 methanol, C-3 propanol and C-5 amyl alcohol.

The amino acid solutions (0.03M) were spotted individually onto the 7 X 4 1/2 inch rectangles of Whatman #1 Chromatography paper. The paper was stapled in the shape of a cylinder and placed in a chromatography chamber. The solvent was allowed to rise about 3 1/2 inches or to about 1 cm. from the top. The paper was then removed, dried in an oven and then sprayed with ninhydrin (0.25% in butanol). After drying in the oven (105 ^{o}C) the spots corresponding to the individual amino acids can be seen. R_f values are then calculated (Table 2).

Computer analysis was done on a IBM compatible computer with a hard disk using Number Cruncher Statistical Software and its Experimental Design package (N.C.S.S., Kayesville, Utah 84037).

RESULTS AND DISCUSSION

Table 1 lists the central composite (3 factors) design. Experiments 1-8 constitute a 2^3 factorial design. Experiments 9-11 and 18-20 constitute a 6 fold duplication of the center point. Experiments 12-17 constitute the star design. The spacing (1.73) is chosen so that the design is rotatable. Table 2 contains the raw R_f data for the 5 amino acids (arginine, leucine, histidine, threonine and tryptophan).

Tables 3 to 5 give the relevant statistical output. The results are all corrected for pure error because of the duplication of the center point. The mean squares calculated by

Table 1 Experimental Design

Experiment Number	Experimental Level		
	Methanol	Propanol	Amyl Alcohol
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	+1	-1	+1
7	-1	+1	+1
8	+1	+1	+1
9	0	0	0
10	0	0	0
11	0	0	0
12	-1.73	0	0
13	+1.73	0	0
14	0	-1.73	0
15	0	+1.73	0
16	0	0	-1.73
17	0	0	+1.73
18	0	0	0
19	0	0	0
20	0	0	0

-1 = 15%, +1 = 25%, 0 = 20%, -1.73 = 11.4%, +1.73 = 28.7%

$\frac{X - 20}{5}$ = coded level

the sequential sum of squares divided by the degrees of freedom are used to calculate experimental F ratios. The probability corresponding to the F ratio is also shown. A probability significance level of $\alpha = 0.10$ is used to test if the ratio is significant. This means if a probability is greater than 0.10 then that term is discarded.

Table 2 Experimental R_f Values

Experiment Number	Amino Acid Arginine	Histidine	Leucine	Threonine	Tryptophan
1	0.719	0.640	0.813	0.725	0.753
2	0.675	0.552	0.831	0.649	0.708
3	0.580	0.477	0.830	0.596	0.705
4	0.539	0.461	0.854	0.596	0.680
5	0.528	0.449	0.775	0.584	0.663
6	0.483	0.371	0.803	0.551	0.607
7	0.404	0.337	0.753	0.494	0.589
8	0.395	0.254	0.798	0.497	0.537
9	0.573	0.460	0.837	0.596	0.674
10	0.565	0.452	0.842	0.633	0.689
11	0.568	0.438	0.836	0.601	0.701
12	0.567	0.483	0.775	0.573	0.674
13	0.486	0.395	0.825	0.565	0.633
14	0.621	0.559	0.814	0.655	0.695
15	0.446	0.339	0.808	0.525	0.588
16	0.678	0.583	0.889	0.722	0.767
17	0.358	0.284	0.761	0.466	0.557
18	0.565	0.464	0.840	0.623	0.702
19	0.565	0.456	0.848	0.630	0.710
20	0.568	0.460	0.840	0.632	0.700

Table 3 summarizes two analyses for each amino acid, the model to be chosen (linear, quadratic and cross product terms) and the solvent effects (methanol, propanol, amyl alcohol). In only two cases (Threonine and Tryptophan) is there a significant term and that is the cross product term (0.1762 and 0.2783). There are two marginal cases where the probability is close to 0.1 (quadratic and cross terms for histidine).

Table 3 Statistical Date (ANOVA)

ANOVA Report (corrected using Pure error)

Amino Acid	Source	DF	Sequential Sum-Squares	Mean Square	F ratio	Probability
Arginine	Regression	9	0.1687163	1.874×10^{-2}	1899	0.000
	Linear	3	0.163069	5.435×10^{-2}	5509	0.000
	Quadratic	3	4.84×10^{-3}	1.61×10^{-3}	163.5	0.000
	Cross	3	8.06×10^{-4}	2.687×10^{-4}	27.24	0.0016
	Methanol	4	7.736×10^{-3}	1.934×10^{-3}	196	0.000
	Propanol	4	4.642×10^{-2}	0.0116063	1176	0.000
	Amyl Alcohol	4	0.11647	2.911×10^{-2}	2951	0.000
Histidine	Regression	9	0.1770	1.967×10^{-2}	229	0.000
	Linear	3	0.1750	5.83×10^{-2}	678	0.000
	Quadratic	3	9.878×10^{-4}	3.29×10^{-4}	3.83	0.0912
	Cross	3	1.045×10^{-3}	3.48×10^{-4}	4.05	0.0831
	Methanol	4	1.379×10^{-2}	3.45×10^{-3}	40.11	0.0005
	Propanol	4	5.40×10^{-2}	1.35×10^{-2}	157	0.000
	Amyl Alcohol	4	0.11048	2.76×10^{-2}	321	0.000
Leucine	Regression	9	2.138×10^{-2}	2.37×10^{-3}	130	0.000
	Linear	3	1.616×10^{-2}	5.39×10^{-3}	294	0.000
	Quadratic	3	4.41×10^{-3}	1.47×10^{-3}	80	0.001
	Cross	3	8.10×10^{-4}	2.70×10^{-4}	14.76	0.0064
	Methanol	4	6.003×10^{-3}	1.500×10^{-3}	82	0.0010
	Propanol	4	2.206×10^{-3}	5.52×10^{-3}	30.1	0.0011
	Amyl Alcohol	4	1.484×10^{-2}	3.71×10^{-3}	203	0.0000

(continued)

Table 4 gives the most important data to the researcher. The coefficients (parameter estimates) are given for each model with the probability given in brackets behind each term. Thus the R_f of arginine can be calculated using the following equation using coded solvent percentages. X_1, X_2 and X_3 are the coded percentages for methanol, propanol and amyl alcohol. All of the

Table 3 Continued

Amino Acid	Source	DF	Statistical Sum-Squares	Mean Square	F ratio	Probability
Threonine	Regression	9	8.59×10^{-2}	9.55×10^{-3}	35.2	0.0005
	Linear	3	7.846×10^{-2}	2.61×10^{-2}	96.5	0.0001
	Quadratic	3	5.46×10^{-3}	1.82×10^{-3}	6.72	0.0332
	Cross	3	2.013×10^{-3}	6.71×10^{-4}	2.48	0.1762
	Methanol	4	7.045×10^{-3}	1.76×10^{-3}	6.5	0.0324
	Propanol	4	2.481×10^{-2}	6.20×10^{-3}	22.9	0.0021
	Amyl Alcohol	4	5.717×10^{-2}	1.429×10^{-2}	52.75	0.0003
Tryptophan	Regression	9	7.212×10^{-2}	8.013×10^{-3}	49.71	0.0002
	Linear	3	6.345×10^{-2}	2.11×10^{-2}	131	0.000
	Quadratic	3	7.831×10^{-3}	2.61×10^{-3}	16.19	0.0052
	Cross	3	8.30×10^{-4}	2.768×10^{-4}	1.72	0.2783
	Methanol	4	7.595×10^{-3}	1.898×10^{-3}	11.78	0.0093
	Propanol	4	1.726×10^{-2}	4.31×10^{-3}	26.77	0.0014
	Amyl Alcohol	4	4.987×10^{-2}	1.246×10^{-2}	77.35	0.0001

probabilities are less than 0.10 so all terms are included in the model

$$R_f = 0.5673 - .0199\ X_1 - .0565\ X_2 - 0.0898\ X_3 - 0.0109\ X_1^2 - 0.00853\ X_2^2 - 0.0137\ X_3^2 + 0.00487\ X_1X_2 + 0.00388\ X_1X_3 + 0.00788\ X_2X_3$$

In histidine the C_2^2 and C_2C_3 terms are rejected. In leucine the C_2 term is rejected. In threonine the C_1 term, C_3^2 term, C_1C_3 term and C_2C_3 terms are rejected. Lastly in tryptophan all 3 cross terms (C_1C_2, C_1C_3, C_2C_3) are rejected. These results agree with the conclusions drawn in Table 3.

These second order equations can be differentiated and their first derivatives set to zero. The solution of these equations lead to the coordinates of the stationary point. The coordinates

Table 4 Parameter Report (using Pure Error) for Each Amino Acid

Parameter	Amino Acid				
	Arginine	Histidine	Leucine	Threonine	Tryptophan
Intercept	0.5673	0.4550	0.8405	0.61916	0.69599
Methanol (C1)	-1.99×10^{-2} (0.000)	-2.98×10^{-2} (0.0001)	$+1.312 \times 10^{-2}$ (0.0001)	-8.5687×10^{-3} (0.1059)	-1.7799×10^{-2} (0.0033)
Propanol (C2)	-5.6468×10^{-2} (0.000)	-6.1748×10^{-2} (0.000)	-1.0997×10^{-3} (0.3805)	-3.93899×10^{-2} (0.0000)	-2.8966×10^{-2} (0.0004)
Amyl Alcohol (C3)	-8.9849×10^{-2} (0.000)	-8.839×10^{-2} (0.000)	-3.1349×10^{-2} (0.000)	-6.3127×10^{-2} (0.000)	-5.815×10^{-2} (0.000)
$C1^2$	-1.0874×10^{-2} (0.000)	-4.9329×10^{-3} (0.0889)	-1.402×10^{-2} (0.000)	-1.633×10^{-2} (0.0049)	-1.362×10^{-2} (0.0081)
$C2^2$	-8.5356×10^{-3} (0.0001)	-1.59162×10^{-3} (0.5268)	-1.0346×10^{-2} (0.0002)	-9.315×10^{-3} (0.0679)	-1.76319×10^{-2} (0.0027)
$C3^2$	-1.37145×10^{-2} (0.000)	-6.770×10^{-3} (0.0341)	-5.6682×10^{-3} (0.0033)	-7.9785×10^{-3} (0.1101)	-1.0782×10^{-2} (0.0200)
C1*C2	$+4.875 \times 10^{-3}$ (0.0071)	8.375×10^{-3} (0.0510)	$+5.125 \times 10^{-3}$ (0.0195)	$+1.4 \times 10^{-2}$ (0.0528)	$+3.00 \times 10^{-3}$ (0.5335)
C1*C3	$+3.875 \times 10^{-3}$ (0.0175)	-7.125×10^{-3} (0.0818)	$+6.125 \times 10^{-3}$ (0.0098)	$+5.75 \times 10^{-3}$ (0.3883)	-4.75×10^{-3} (0.3384)
C2*C3	$+7.875 \times 10^{-3}$ (0.009)	3.124×10^{-3} (0.3843)	-6.125×10^{-3} (0.0098)	$+4.75 \times 10^{-3}$ (0.4733)	-8.50×10^{-3} (0.1168)

The number in brackets is the probability.

Table 5 Optimum Conditions (Stationary points)

Amino Acid	Methanol	Propanol	Amyl Alcohol	Type
Arginine	-3.509	-6.973	-5.773	Maximum
Histidine	16.9248	12.903	-12.455	Saddlepoint
Leucine	-0.088125	0.90194	-3.300	Maximum
Threonine	-5.1939	-8.1195	-8.244	Maximum
Tryptophan	-0.2322	-0.22486	-2.5568	Maximum

of these points are given in Table 5. In 4 out of the 5 cases a maximum is observed. In the fifth case a saddle point is observed. Coordinates outside the normal range of 0 to 100% are not possible, i.e. for Arginine the maximum is at 2.45% Methanol, -14.86% propanol and -8.86% amyl alcohol. Tryptophan has a maximum at 18.84% methanol, 18.87% propanol and 7.21% amyl alcohol which is possible.

This study has resulted in the following conclusions.

1) Second order equations relating R_f to solvent composition of the three alcohols are given.
2) These equations are unique to each amino acid and in some cases constants for selected terms are not significant, i.e. histidine, threonine and tryptophan and thus the shapes of the response surfaces are different.
3) Not all optima are in the real domain and thus one only sees the ridges on one side of the curve. All optima are unique for each amino acid.
4) Amyl alcohol has a greater effect on the R_f than propanol which is greater than ethanol.
5) The best separation $\wedge$ R_f can be calculated and optimized also because the equations for each component amino acid are available.

Knowledge of the equation for the response surface will enable R_f to be calculated for future solvent mixtures.

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