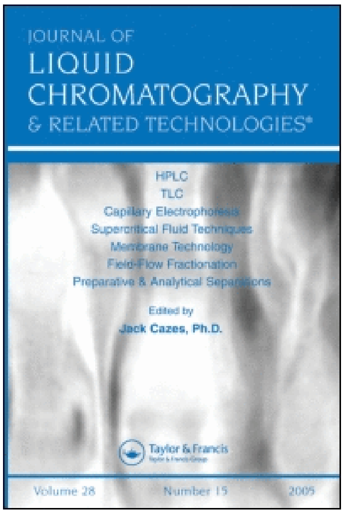

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OPTIMIZATION OF SOLVENT COMPOSITION FOR HIGH PERFORMANCE THIN-LAYER CHROMATOGRAPHY

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ABSTRACT

The methodology of mixture experiments has been applied to optimize the multicomponent solvent composition for high performance thin-layer chromatography (HPTLC). In this study, a mixture of five solvents for the mobile phase was maximized for the separation of twelve structurally related B(a)P metabolites on normal phase HPTLC plates. Volume fractions (rather than absolute quantities) of the mobile phase solvents were constrained by upper bounds by considering solvent strength. These restrictions defined an experimental region that is an irregular polyhedron formed from a truncated 4-dimensional simplex. The optimization of the expected separation response over the experimental region was based on a second-order Scheffé polynomial estimated from twenty-five experimental runs. Six functions for measuring separation based on R_f values were evaluated. A new response function is proposed that overcomes the difficulties of previously published criteria.

INTRODUCTION

In liquid chromatography, finding the "best" solvent composition for the mobile phase is often a major task. In particular, complexities are introduced in assessing multicomponent solvent strengths and solvent selectivities. In addition, solvent proportions in the mobile phase vary from 0 to 1, and the sum of the proportions for all components is 1. These restrictions require a different experimental strategy than the normal factorial and response surface experiments that use independent factors. The appropriate experimental method is to use mixture designs. Mixture experimental designs have been applied to optimize the mobile phase in high performance liquid chromatography (1-7). However, a recent review (8) noted that most mobile phase optimization procedures are limited to three or less components and most methodologies do not account for components that have constraints on the upper limits of their proportions.

In this study, the methodology of mixture experiments (9) was used to find the optimal mobile phase composition of five solvents for high performance thin-layer liquid chromatography (HPTLC). Benzo(a)pyrene and its oxygenated metabolites were chosen as test compounds because of their importance as toxic compounds and the problems of separating these structurally related derivatives. HPTLC was used to separate these compounds instead of the conventional thin layer chromatography (10,11) because HPTLC provides a higher resolution efficiency and shorter developing time.

The conclusion from this study indicated that the mixture of 63% hexane, 30% benzene, and 7% isopropyl alcohol would give a reasonable separation of the twelve B(a)P related compounds. This result may not be unique and other possibilities can be evaluated by using the response surface model estimated from the mixture experiment. Contours of the response for various combinations of the five solvents gave a better understanding of the effect of the solvents on HPTLC separation.

THEORY

In a mixture experiment, the response from a mixture of q components is a function of the proportions X_1, X_2, ..., X_q of the components in the mixture rather than the absolute quantities. The proportions X_j must satisfy the constraints

$$0 \leq X_j \leq 1 \quad \text{and} \quad \sum_{j=1}^{q} X_j = 1. \qquad [1]$$

In addition, chemical constraints may also be needed such as solvent strength and solvent selectivity. These properties may restrict the lower and upper bounds on the proportions X_j.

$$L_j \leq X_j \leq U_j \qquad [2]$$

For example, the values of L_j and U_j were chosen in this experiment based on the estimated Snyder's solvent strength (ϵ) (12,13) and the elution behavior of the samples as observed in preliminary experiments. Care must be taken that the bounds define a consistent constrainted region (5). The restrictions placed on the experimental region by conditions [1] and [2] make it impossible to vary the components independently of each other and define a complex geometry that is an irregular polyhedron formed from a truncated simplex (14). Conditions [1] define a (q-1)-dimensional simplex and condition [2] truncate this simplex.

The most common experimental designs for a constrained mixture problem are extreme-vertices designs (15). These designs are made up of those points which lie on the intersections of the constraint boundaries. Frequently, additional experimental runs must be added to estimate all the coefficients in the approximating response model and estimate experimental error. These extra runs are usually chosen with the aid of a computer algorithm especially when more than three mixture components are involved.

Modeling the response of a mixture experiment is another area of departure from the usual response surface models. Frequently, a conventional response surface model will represent the expected response with a low-order polynomial derived from a Taylor Series. Experience has shown that linear and quadratic models are the most useful to represent the response data, and on some occasions additional cubic terms are included. For mixture models, these polynomials will have fewer terms because the component proportions sum to 1. In particular, a linear polynomial will not have an intercept term and a second-order polynomial will have neither an intercept term nor pure quadratic terms (ie, X^2 terms).

Scheffé (16) proposed the following canonical second-order polynomial model for the expected response by applying the restrictions in [1]:

$$E(\text{response}) = \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta q X_q + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \ldots + \beta_{q-1,q} X_{q-1} X_q \quad [4]$$

The absence of an intercept term requires that special attention must be paid to the analysis method and interpretation of the results. The linear coefficients in the canonical polynomial are interpreted as the expected response to the pure component. For example, if $X_1 = 1$ and $X_2 = X_3 = \ldots = X_q = 0$ then E(response) $= \beta_1$. Coefficients for the mixed terms that represent beneficial effects are called synergistic and those coefficients representing harmful effects are called antagonistic effects.

Testing for significant coefficients in mixture models also departs from standard regression analysis due to the absence of an intercept term. For example, testing the hypothesis that the response does not depend on the mixture components is equivalent to testing that the individual linear coefficients are equal (ie, not zero) and the higher-order coefficients are zero. Additional information on the analysis of models for mixture experiments can be found in the literature (9,17,18).

The separation response from each experimental run was a distance measure based on the twelve measured R_f values. Initially, the all pairwise distances and all pairwise inverse distances suggested by Gonnord et. al. (19) were used as separation responses. However, these two measures proved unsatisfactory because a maximum distance could be obtained when all R_f values except one had a zero value and the one non-zero R_f value was large. Two additional distance measures also proved unsatisfactory: the all pairwise distances measured by the logarithm of the absolute differences and the adjacent pair distances. The final response used to measure separation was a sum of two terms. The first term measured the difference of the R_f values from an ideal separation on the unit line and the second term measured a property of uniformity represented by a statistic called kurtosis.

EXPERIMENTAL

All solvents were high purity B and J Brand (Burdick and Jackson Laboratory, Muskegon, MI). Benzo(a)pyrene was purchased from the Aldrich Chemical Co. (Milwaukee, WI) and was used without further purification. Oxygenated derivatives of benzo(a)pyrene, as listed in Table 1, were obtained from the NCI Chemical Repository at the IIT Research Institute (Chicago, IL) and were used as received. Solutions of these compounds were prepared at concentrations of 0.5 to 1.0 mg/ml by dissolving weighed amounts in methylene chloride or ethyl acetate.

Precoated high performance silica gel plates with UV-254 indicator (HP-KF, 10 x 10 cm, 200 μm thickness) were purchased from

TABLE 1. Benzo(a)Pyrene and Oxygenated Derivatives to be Separated by HPTLC.

COMPOUND	ABBREVIATION
1: Benzo(a)pyrene	A: B(a)P
2: 3-Hydroxybenzo(a)pyrene	B: 3-OH-BaP
3: 6-Hydroxybenzo(a)pyrene	C: 6-OH-BaP
4: 8-Hydroxybenzo(a)pyrene	D: 8-OH-BaP
5: 9-Hydroxybenzo(a)pyrene	E: 9-OH-BaP
6: 10-Hydroxybenzo(a)pyrene	F: 10-OH-BaP
7. Benzo(a)pyrene-1,6-dione	G: BaP-1,6-dione
8. Benzo(a)pyrene-3,6-dione	H: BaP-3,6-dione
9. Benzo(a)pyrene-7,8-dione	I: BaP-7,8-dione
10. Benzo(a)pyrene-6,12-dione	J: BaP-6,12-dione
11. Benzo(a)pyrene-trans-9,10-dihydrodiol	K: BaP-diol
12. Benzo(a)pyrene-7,8-dihydro-epoxide	L: BaP-epoxide

Whatman, Inc. (Clifton, NJ) and developed in an ascending direction in the tank, which was pre-equilibrated with mobile phase by a saturation pad. Under a stream of nitrogen, an aliquot of 0.2 to 1.0 μl of solution was applied to the plate with a microsyringe. The spots at the origin generally ranged from 2-4 mm and grew to 4-7 mm after developing. A typical development required approximately 15 minutes for the solvent front to reach 75 mm from the origin. Spots were visible under both 360 nm and 254 nm UV lamps in a light shielding box.

Four commonly used mobile phase solvents with medium to high solvent strengths (ϵ) were selected from Snyder's seven selectivity groups (20). These four solvents (benzene, methylene chloride, ethyl acetate, and isopropyl alcohol) were chosen from four different groups to provide maximum selectivity. Preliminary tests were made to estimate the approximate solvent strenght of mobile phase solutions for reasonable overall separations. Solvents with high ϵ such as ethyl acetate and isopropyl alcohol were tested in combination with hexane, while solvents with medium ϵ such as benzene and methylene chloride were tested both as pure solvent and in combination with hexane. For example, the following mobile phases provided approximately equal overall visual separation of the twelve compounds : 100% benzene (ϵ = 0.26), 90% methylene chloride in hexane (ϵ = 0.33), 60% ethyl acetate in hexane (ϵ = 0.41), and 7% isopropyl alcohol in hexane (ϵ = 0.49). Using Snyder's solvent scale as a guide, the oxygenated derivatives of B(a)P should not migrate with mobile phases of extremely low ϵ (< 0.08) such as 7% benzene in hexane. Mobile phases with very high ϵ (> 0.54) such as a combination of 33% methylene chloride, 60% ethyl acetate, and 7% isopropyl alcohol should cause all the twelve compounds to migrate close to the solvent front. These preliminary trials indicated that volume range restrictions given in Table 2 should be appropriate to evaluate multicomponet mobile phases. The methodology applied in this study would be the same for any set of solvents with restricted volume percent ranges.

TABLE 2. Range of Proportions and Snyder's Solvent Strength for the Five Solvents in the Mobile Phase.

Solvent	Solvent Strength on Silica Gel[a]	Selectivity Group[b]	Range of Volume %	Solvent Strength Range[c]
H: Hexane	0.01	---	0 - 93	0.00 - 0.01
B: Benzene	0.26	VII	0 - 100	0.00 - 0.26
M: Methylene Chloride	0.34	V	0 - 90	0.00 - 0.33
E: Ethyl Acetate	0.46	VI	0 - 60	0.00 - 0.41
I: Isopropyl Alcohol	0.66	II	0 - 7	0.00 - 0.49

a) ϵ (silica) = 0.8ϵ (alumina) (13).
b) Reference (20).
c) Estimated as binary solvent strength in combination with hexane (12).

The restrictions on the five solvents define a consistent constraint experimental region. The geometry of the experimental region is a polyhedron formed from a truncated 4-dimensional simplex.

The optimization of an expected separation response over the experimental region was approximated by a second-order Scheffé's polynomial given in [4]. This approximating model has 15 coefficients to be estimated and therefore any experiment requires at least 15 experimental runs. An additional 5 experimental runs were included to estimate experimental error. These initial 20 experimental runs were chosen in three stages.

Stage 1 selected 11 experimental runs using the extreme vertices algorithm XVERT1 (21). This algorithm actually generated 12 runs, but one run was rejected because the resulting solution would have had solvent strength that was considered to be too low. Stage 2 selected the centroid or average value of the 11 extreme value vertices. Stage 3 selected the remaining 8 runs by a computer search (22,23) over the average of all possible pairs (66 pairs) of the 12 runs in stages 1 and 2. This computer search used a variance criterion called D-optimality (24,25). D-optimality selects designs that minimize both the average variance of predicted responses and the volume of the confidence ellipsoid for the coefficients in the approximating model.

SEPARATION RESPONSE

The response of an individual metabolite component on an HPTLC plate was measured by the fraction of the migration distance to the solvent front, L, by the R_f values

$$R_f = Z/L$$

where Z is the migration distance measured at the center of the spot. All experimental runs had a solvent front of L = 75mm and an eluent time of about 15 minutes.

Separation measurements of q-components for HPTLC are defined as distance functions of the R_f values. Four distance measures are suggested from the literature (1,19) may be used as optimum separation functions for HPTLC.

1. Maximize Overall Distance:

$$D1 = \{ \sum_{j=1}^{q-1} \sum_{k=j+1}^{q} [(R_f)_j - (R_f)_k]^2 \}^{1/2}$$

2. Minimize Inverse Distance:

$$D2 = \{ \sum_{j=1}^{q-1} \sum_{k=j+1}^{q} 1 / [(R_f)_j - (R_f)_k]^2 \}^{1/2}$$

3. Maximize a Chromatography Response Function:

$$D3 = \sum_{j=1}^{q-1} \sum_{k=j+1}^{q} \ln(|(R_f)_j - (R_f)_k|)$$

4. Maximize Adjoining Distances:

$$D4 = \{ \sum_{j=1}^{q} [(R_f)_j - (R_f)_{j+1}]^2 \}$$

These four responses were calculated for the twenty experimental runs of the mixture experiment. The coefficients of Scheffé's polynomial were estimated by least squares methods for each response. From these fitted models, the optimum separation was determined by a computer search over possible values of the five solvent factors. An additional experimental run was then performed to verify the predicted optimum. The result was very disappointing because only B(a)P moved while the other eleven components remained at the base line.

Investigation of the properties of the four response functions showed that a few eccentric spots can inflate the distance measure. This property can be illustrated by noting that the ideal separation of four components would have R_f values of (0,1/3,2/3,1) and the four distance measures would have values of D1 = 1.49, D2 = 3.61, D3 = -4.11, and D4 = 0.58. Now consider the four R_f values (0,0,0,1), the four distance measures would have the values D1 = 1.73, D2 = 1.73 (eg., recall D2 is to be minimized), D3 = 0.00, and D4 = 1.00. For D2 and D3, terms that involve equal R_f values were deleted or set equal to zero. All four distance measures give better results for (0,0,0,1) than the ideal case.

To overcome the problem of one point dominating the distance measure, a new measure was investigated that measured the difference of R_f values from the ideal case. The ideal separation would have q-components equally spaced on the unit interval [0,1]. The ideal value for the j-th ordered R_f value would be (j-1)/(q-1). If the R_f values are ordered in ascending order a new optimum separation measure would be to minimize:

$$D5 = \{ \sum_{j=1}^{q} [(R_f)_j - (j-1)/(q-1)]^2 \}^{1/2}$$

Using D5 as a separation measurement, the optimum condition was calculated to be at 30% benzene, 40% hexane, and 30% isopropyle alcohol. These conditions were used for experimental run 22. The results showed good separation but indicated a problem with the D5 response measurement. This problem is again illustrated with 4-components for two sets of R_f values (1/6,1/6,5/6,5/6) and (1/6,1/2,5/6,5/6) for which D5 has the same value (D5 = 1/3). Although both sets of values have the same D5 values, the second set is more spread out than the first set.

Response measure D5 was improved by adding a term that measured spread represented by the standardized fourth central moment of the R_f values. The first moment and r = 2, 3, and 4 higher central moments are defined for q-components as:

$$M_1 = \sum_{j=1}^{q} (R_f)_j/q \quad \text{and } M_r = \sum_{j=1}^{q} [(R_f)_j - M_1]^r/q \quad r = 2,\ 3,\ 4.$$

The standardized fourth central moment is defined by:

$$b_2 = M_4/M_2{}^2.$$

This standardized fourth central moment is referred to as kurtosis in the statistical literature and has been used to characterize probability distributions. For q-components with R_f values equally spaced in the unit interval, the exact value of b_2 is:

$$B_2 = 3(3q^2 - 7)/[5(q-1)(q+1)].$$

Separation measurement D6 incorporates both a deviation from the ideal spacing as well as a spread factor.

$$D6 = \{ \sum_{j=1}^{q} [(R_f)_j - (j-1)/(q-1)]^2 + (b_2 - B_2)^2 \}^{1/2}.$$

Ranking the separations for the 22 plates using the D6 response measurements agreed very well with the rank of the separations by visual inspection. The best condition using the first twenty experimental runs predicted a solution of 10% benzene, 86% hexane, and 4% isopropyl alcohol. This solution was replicated in the 23rd and 24th experimental runs. The separation results were considered to be only marginally good. An improved mobile solvent solution for separation was found by using all 24 experimental runs to estimate the prediction equation. The final solution was predicted for 30% benzene, 63% hexane, and 7% isopropyl alcohol which gave the best separation of all experimental runs.

RESULTS AND DISCUSSION

The proportions of the mobile phase solvents and the resulting R_f values are tabulated in Table 3. The data are ranked from the lowest to highest D6 measurements. The method of least squares was used to fit the twenty-five D6 separation measurements to a second-order Scheffé's polynomial as a function of the five solvent components. Interaction terms were eliminated that were not significantly different than zero at the 15% significance level using the stepwise elimination method (26). The final prediction equation with the standard deviations of the coefficients in parentheses is:

$$\begin{aligned} D6 = {} & \underset{(1.05)}{7.72H} + \underset{(0.81)}{2.90B} + \underset{(0.90)}{0.68M} + \underset{(1.36)}{6.64E} + \underset{(12.65)}{29.49I} \\ & - \underset{(4.09)}{10.31HB} - \underset{(4.47)}{13.86HE} - \underset{(26.70)}{116.25HI} + \underset{(4.95)}{7.68BM} + \underset{(20.72)}{38.72MI} \end{aligned}$$

where H, B, M, E, and I are proportions of the five solvents.

This response surface equation explains 78% of the total variation in the data. The estimated standard deviation for an individual measurement is S = 1.10 with an associated 10 degrees of freedom. This large standard deviation indicates that about five mixtures of the mobile phase solvents are equivalent to the mixture with the lowest predicted value (ie, D6 = 0.72). For example, the first five mixtures listed in Table 3 may be equally adequate for mobile phase solvents.

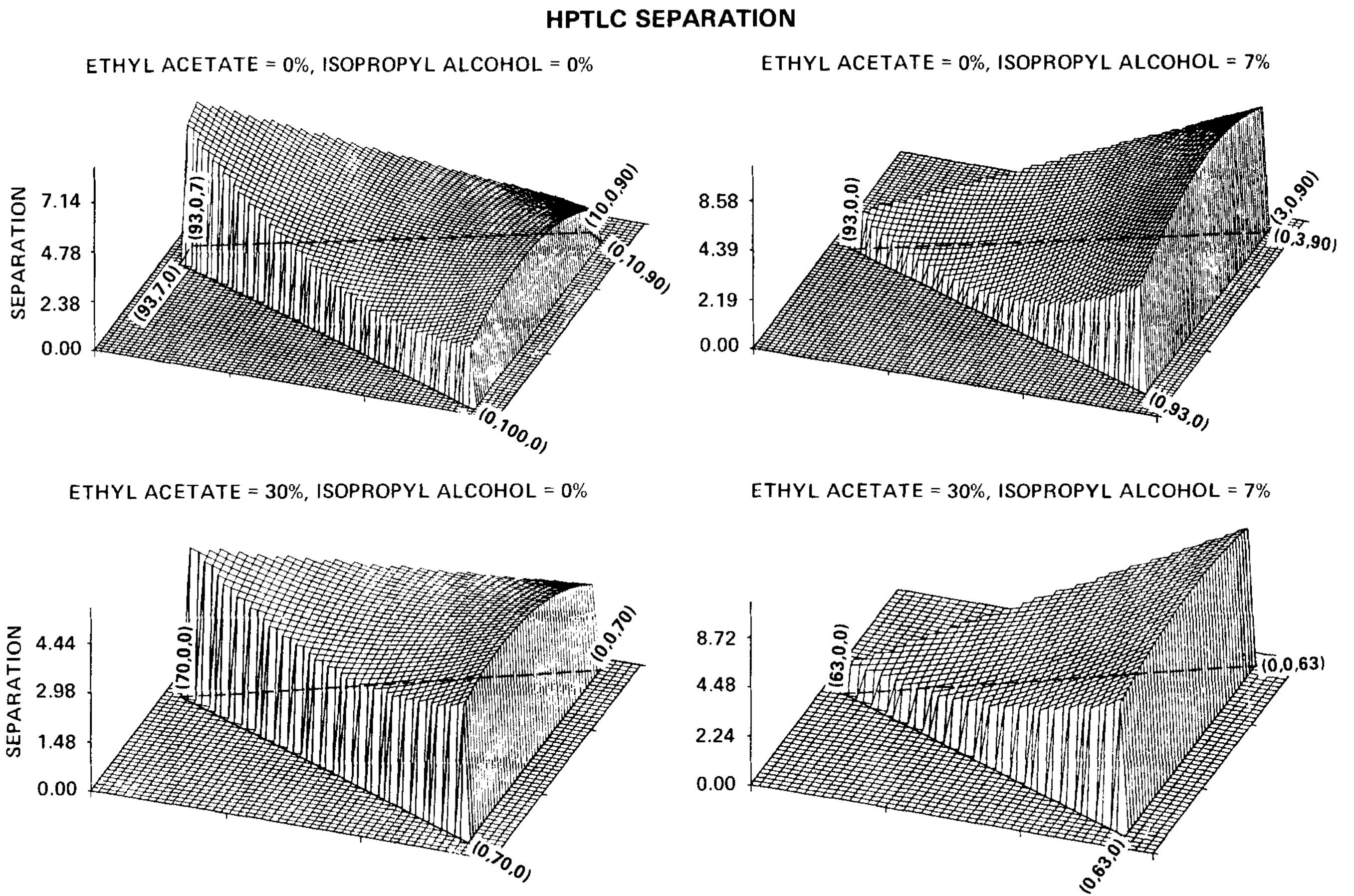
It is important to remember the restrictions on the proportions when using the response surface equation. For example, the equation indicates that the lowest value of D6 would occur with 100% methylene chloride (eg, M = 1.0, H = B = E = I = 0.0) but the upper bound on methylene chloride is 90% and the remaining volume must include some of the other four solvents.

Terms with positive coefficients are considered to have antagonistic effects while those with negative coefficients have synergistic effects because the optimum criteria is to minimize D6. Therefore, the mixtures of hexane and benzene, hexane and ethyl

TABLE 3. R_f Values for HPTLC Mixture Experiment.

		Solvents Proportion (Vol/Vol %)						Rf Values x 100											
Rank	Run	H	B	M	E	I	D6	A	B	C	D	E	F	G	H	I	J	K	L
1	25	63.0	30.0	0.0	0.0	7.0	1.30	76	41	55	38	39	45	31	34	39	50	25	65
2	2	0.0	10.0	90.0	0.0	0.0	1.38	79	32	46	23	26	47	7	11	18	17	0	52
3	7	93.0	0.0	0.0	0.0	7.0	1.46	67	27	33	24	26	29	19	13	0	0	0	51
4	3	10.0	0.0	90.0	0.0	0.0	1.50	77	27	42	19	19	43	5	7	13	10	0	50
5	22	40.0	30.0	0.0	30.0	0.0	1.60	69	46	53	43	42	59	33	24	35	43	5	54
6	17	51.5	0.0	45.0	0.0	3.5	1.84	76	36	45	32	33	44	30	41	45	49	6	64
7	18	66.5	0.0	0.0	30.0	3.5	1.97	71	53	59	51	53	59	39	31	39	47	9	60
8	15	46.5	50.0	0.0	0.0	3.5	2.17	71	39	46	33	33	43	33	37	37	49	4	61
9	1	0.0	100.0	0.0	0.0	0.0	2.65	80	23	39	19	20	37	7	7	8	13	3	43
10	23	86.0	10.0	0.0	0.0	4.0	2.75	67	22	26	19	21	25	19	16	13	29	0	47
11	24	86.0	10.0	0.0	0.0	4.0	2.78	68	21	26	19	19	25	19	15	13	30	0	47
12	13	0.0	55.0	45.0	0.0	0.0	3.51	73	21	32	13	14	31	3	3	4	5	0	27
13	5	40.0	0.0	0.0	60.0	0.0	3.56	66	52	57	51	51	55	41	39	46	49	12	59
14	16	25.0	0.0	45.0	30.0	0.0	3.95	73	58	62	55	55	65	41	47	54	55	9	64
15	19	48.0	0.0	45.0	0.0	7.0	4.18	77	53	60	51	53	57	51	54	59	61	15	70
16	11	33.0	0.0	0.0	60.0	7.0	4.47	71	65	68	64	66	67	57	53	61	62	31	65
17	6	0.0	93.0	0.0	0.0	7.0	4.87	74	52	61	51	52	59	53	55	59	62	13	67
18	14	0.0	70.0	0.0	30.0	0.0	4.89	71	56	62	54	55	63	46	47	54	56	9	65
19	20	1.5	1.5	90.0	0.0	7.0	4.93	84	67	73	61	63	73	63	69	75	69	23	80
20	8	0.0	3.0	90.0	0.0	7.0	5.07	91	70	79	67	69	77	70	76	81	77	29	85
21	4	0.0	40.0	0.0	60.0	0.0	5.29	71	59	64	58	59	64	52	52	58	59	21	63
22	10	0.0	33.0	0.0	60.0	7.0	5.54	74	67	70	67	70	71	63	61	66	66	39	69
23	9	3.0	0.0	90.0	0.0	7.0	5.61	80	66	73	63	65	71	66	71	75	72	25	82
24	12	16.0	25.0	33.0	22.0	4.0	5.61	75	63	67	61	63	69	53	57	62	63	17	69
25	21	90.0	0.0	10.0	0.0	0.0	8.53	29	0	0	0	0	0	0	0	0	0	0	0

acetate, and hexane and isopropyl alcohol are beneficial for reducing D6, while the mixtures of benzene and methylene chloride, and methylene chloride and isopropyl alcohol are detrimental for reducing D6. The synergistic effects and antagonistic effects are illustrated by four response surface plots in Figure 1. For example, the surface on the benzene-methylene chloride boundary (ie, the boundary between coordinates (0,B,0) and (0,0,H)) is convexed for all four plots and represents antagonistic effects of the two solvents. The surface on the hexane-benzene boundary (ie,


HPTLC SEPARATION

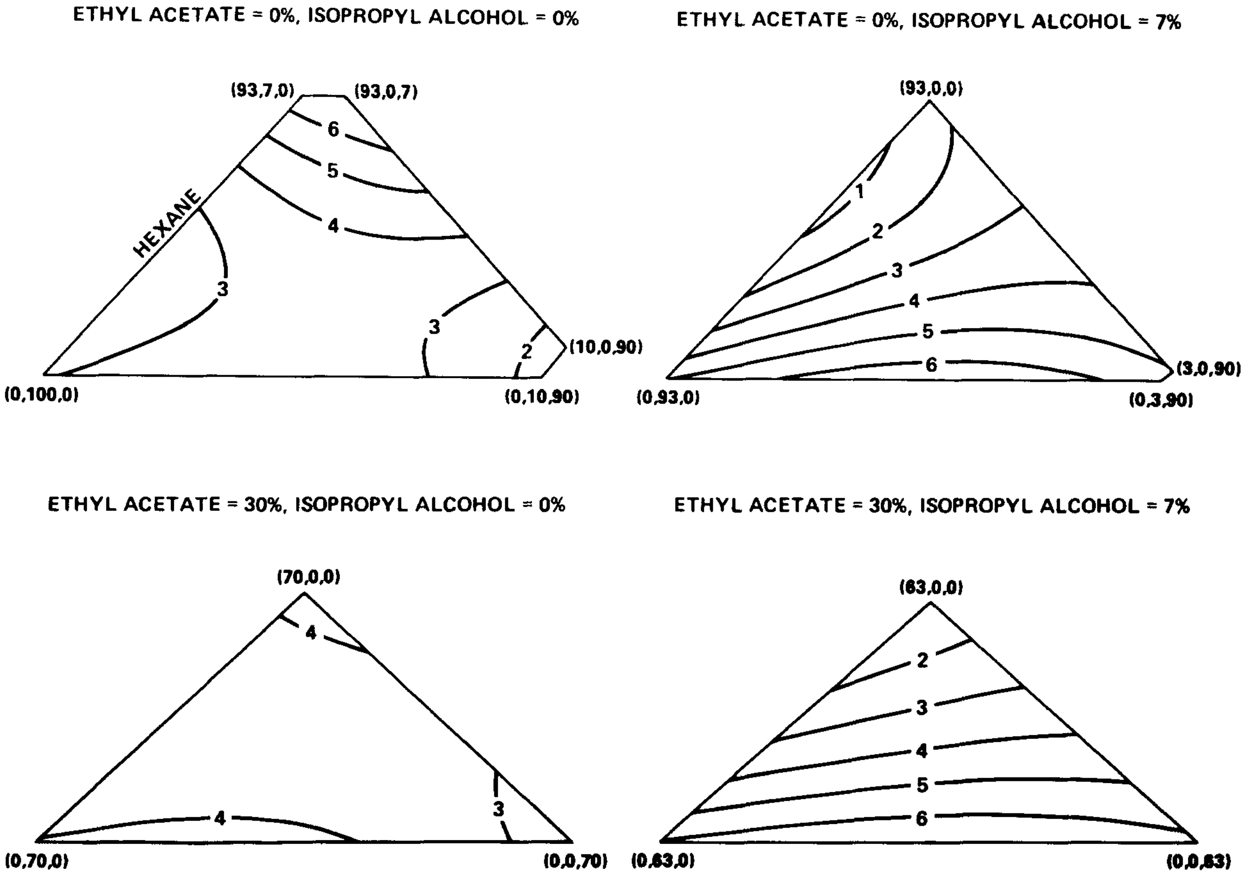
ETHYL ACETATE = 0%, ISOPROPYL ALCOHOL = 0%

ETHYL ACETATE = 0%, ISOPROPYL ALCOHOL = 7%

ETHYL ACETATE = 30%, ISOPROPYL ALCOHOL = 0%

ETHYL ACETATE = 30%, ISOPROPYL ALCOHOL = 7%

Figure 1. Response Surfaces for HPTLC Separation With Coordinates Identified as Volume Percents of (H, B, M)


ETHYL ACETATE = 0%, ISOPROPYL ALCOHOL = 0%

ETHYL ACETATE = 0%, ISOPROPYL ALCOHOL = 7%

ETHYL ACETATE = 30%, ISOPROPYL ALCOHOL = 0%

ETHYL ACETATE = 30%, ISOPROPYL ALCOHOL = 7%

Figure 2. Contours of HPTLC Separation With Coordinates Identified as Volume Percents of (H, B, M)

the boundary between coordinates (H,0,0) and (0,B,0)) is concaved and represents synergistic effects of these two solvents.

After establishing the response surface equation for the separation measurements, a computer search over the experimental region was made for the optimum values of the mobile phase solvents. This search was preformed by incrementing the proportions of the five solvents and examining the prediction equation for the lowest D6 values. This grid search was necessary because of the complex geometry of the experimental region. The search considered 41,469 cases and the lowest D6 value ocurred at 30% benzene, 63% hexane, and 7% isopropyl alcohol which is experimental run 25. The smallest fifty predicted values, $0.72 \leq D6 \leq 1.22$, occurred for the solvent ranges $43\% \leq H \leq 83\%$, $0\% \leq B \leq 50\%$, $0\% \leq M \leq 5\%$, $0\% \leq E \leq 30\%$ and $I = 6\%$ or 7%. Contour plots given in Figure 2 are the two-dimensional projections of the response surfaces in Figure 1. These contours indicate several mixtures of the mobile solvent system that may produce adequate separation.

Although this optimization experiment was designed to find the best solvent composition for separating all twelve test compounds, application to subsets of the test compounds is also applicable. For example, separation of the parent from its metabolites can be easily achieved by all the solvent systems except runs 9 and 20 (see Table 3). Positional isomers such as BaP-diones (compounds G to J) can be separated by runs 17, 23, 24, and 25. Among the dihydroxy derivatives, the suitable solvent systems are runs 1, 25, and 9 with 8-OH-BaP and 9-OH-BaP overlapped.

CONCLUSION

The methodology of mixture experiments was used successfully to optimize the mobile phase solvent system in twenty-five experimental runs for HPTLC. Traditional experimental designs for a five component solvent system would have required many more experiments because of the complex geometry of the experimental region. The final solvent system of 30% benzene, 63% hexane and 7% isopropyl alcohol represents our best solution for the D6 separation measurement. This new separation measurement seems to correspond to visual inspection much better than other separation measurements found in the literature.

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