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# A COMPARISON OF OVERLAPPING RESOLUTION MAPPING WITH IDEAL SEPARATION TO OPTIMIZE THE MOBILE PHASE COMPOSITION FOR HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY\*

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## ABSTRACT

The two data analysis methods, overlapping resolution mapping (ORM) and ideal separation (IS), are compared to optimize the multi-component mobile phase composition for high performance thin-layer chromatography (HPTLC). In this study, the separation of 11 anthraquinone dyes are evaluated using both ORM and IS to select the best mixture of three blends for the mobile phase. The three blends are composed of the four solvents benzene, chloroform, tetrahydrofuran, and hexane with each blend having a Snyder's solvent strength of about  $\epsilon = 0.3$ . Comparing the results for the two data analysis methods showed ORM selected mobile phases that maximized the separation of the nearest pair of compounds while IS selected mobile phases that simultaneously maximized the overall separation for all sample components. Selection of the method for data analysis depends on the objective of the optimization process. Resolution contour maps are generated for both ORM and IS to find regions of optimum mobile phase composition.

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### INTRODUCTION

Overlapping resolution mapping (ORM) has been successfully applied to high performance liquid chromatography (HPLC) (1,2) to optimize the separation of adjacent peaks. ORM uses a single-valued response function of the usual resolution functions,  $R_s$ 's, evaluated for all adjacent peaks from an experimental run. A series of experimental runs are performed for different mobile phase compositions and the maximum value is found by overlapping the contours of resolution function values for all adjacent peaks. In this manner, the separation of peaks can be optimized for different properties (e.g., capacity factors, column plate number, and separation factor) of the solvent system. ORM can also be adapted to optimizing the mobile phase composition of high performance thin-layer chromatography (HPTLC) by defining the resolution function in terms of distances between spots and their corresponding spot widths.

Recently, the four common response functions overall distance, inverse distance, logarithm distance, and adjoining distance were investigated (3) for HPTLC. These commonly used response functions were shown to be severely affected by a few eccentric spots. A new response function called the ideal separation response function was developed which can be used to optimize the overall separation of spot distances. This response function is a sum of an ideal spacing term and an ideal spread term. The ideal separation (IS) data analysis method minimizes the ideal separation response function to find the best overall separation among spots.

The ORM and IS data analysis methods are compared for a statistically designed mixture experiment to optimize the mobile phase for separating 11 anthraquinone dyes. For this experiment, the optimum mobile phase depends on the method of data analysis. Using the ORM method, a mobile phase was identified that maximized the separation between the two nearest adjacent dye spots while using the IS method, a mobile phase was identified that maximized the overall separation of all dye spots.

EXPERIMENTAL

HPTLC was used to separate the anthraquinone dyes and the related compounds listed in Table 1. Compounds A through F were obtained from the US Army Armament Research and Development Command, Aberdeen Proving Ground, MD and were purified as described elsewhere (4,5). Compounds F and G were purchased from Fluka Chemical Co. (Ronkonkoma, NY) and were used without further purification. Compounds H, I, J, and K were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were used as received. All solvents for the mobile phase were high purity (i.e., distilled-in-glass grade) B and J Brand (Burdick and Jackson Laboratory, Muskegon, MI).

Solutions of the dyes were made at concentrations of 2 to 5 mg/mL by dissolving weighed amounts in either methylene chloride or tetrahydrofuran. With a Drummond disposable micro-pipette, 0.5 or 1.0  $\mu\text{L}$  of the tested sample was spotted on a high performance alkylamino-bonded silica gel thin layer chromatography plate (pre-coated HPTLC plates,  $\text{NH}_2\text{-F}_{254}\text{-S}$ , 0.2 mm thickness, E. Merck, Darmstadt, West Germany). The samples were spotted 1 cm from the lower edge of the plate, which was then developed in an ascending

TABLE 1. Anthraquinone Dyes and Related Compounds

COMPOUND ID	CHEMICAL	ABBREVIATION
A	1-methylaminoanthraquinone	MAA
B	1,4-diamino-2,3-dihydroanthraquinone	DDA
C	1,4-diaminoanthraquinone	DAA
D	7H-benz[de]anthracen-7-one	BZA
E	dibenzo[b,def]chrysene-7,14-dione	DBC
F	1,4-di-p-toluenedinoanthraquinone	pTA
G	anthraquinone	A
H	2-aminoanthraquinone	2-AA
I	1-aminoanthraquinone	1-AA
J	1,2-diaminoanthraquinone	1,2-DAA
K	2,6-diaminoanthraquinone	2,6-DAA

direction in a pre-saturated tank. A typical development required approximately 15 minutes for the solvent front to reach 85 mm from the origin. All spots, except anthraquinone, showed an intense characteristic color after development. The anthraquinone was visible as a dark blue spot under a 254 nm UV lamp.

To obtain the widest selectivity differences, mobile phase solvents should be selected from groups as close as possible to the three apexes of Snyder's solvent selectivity triangle (6,7,8). In this study, three solvents benzene, chloroform and tetrahydrofuran with medium solvent strength (e.g.,  $\epsilon = 0.32$ , 0.40, and 0.57) were chosen from groups III, VII, and VIII. A preliminary test showed that an approximate mobile phase solvent strength of  $\epsilon = 0.30$  gave reasonable overall separations of the eleven test compounds. This solvent strength can be achieved by making binary blends of the three solvents with hexane ( $\epsilon = 0.00$ ) which is a strength adjusting solvent for normal phase chromatography. The volume fraction of the three binary blends are given in Table 2.

TABLE 2. Volume Fraction of Solvents in the Mobile Phase.

MIXTURE NUMBER	BLEND RATIO <sup>1</sup> (X <sub>1</sub> /X <sub>2</sub> /X <sub>3</sub> )	MOBILE PHASE COMPOSITION <sup>2</sup> (BZ/CLF/THF/HX)
1.	1 / 0 / 0	94 / 0 / 0 / 6
2.	0 / 1 / 0	0 / 75 / 0 / 25
3.	0 / 0 / 1	0 / 0 / 53 / 47
4.	1/2 / 1/2 / 0	47 / 37.5 / 0 / 15.5
5.	0 / 1/2 / 1/2	0 / 37.5 / 26.5 / 36.0
6.	1/2 / 0 / 1/2	47 / 0 / 26.5 / 26.5
7.	1/3 / 1/3 / 1/3	31.3 / 25 / 17.7 / 26.0
8.	2/3 / 1/3 / 1/3	63 / 12 / 8.5 / 16.5
9.	1/3 / 2/3 / 1/3	15 / 50.3 / 8.5 / 26.2
10.	1/3 / 1/3 / 2/3	15 / 12 / 35.5 / 37.5

1) X<sub>1</sub> = blend 94/6 (BZ/HX); X<sub>2</sub> = blend 75/25 (CLF/HX); X<sub>3</sub> = blend 53/47 (THF/HX).

2) BZ = benzene (Group VII,  $\epsilon = 0.32$ ); CLF = chloroform (Group VIII,  $\epsilon = 0.40$ ); THF = tetrahydrofuran (Group III,  $\epsilon = 0.57$ ); HX = hexane ( $\epsilon = 0.00$ ).

A mixture experiment (9) was performed to choose the best mixture of the three binary blends for the mobile phase solvent. The mixture region is a 2-dimensional simplex (i.e., a triangle). Ten experimental mixtures were run in the simplex mixture region consisting of mixtures of the binary blends at the three vertices, the three mid-points on the edges, the centroid, and the three mid-points of the rays connecting the vertices and the centroid. The blend ratios of the binary blends are given in Table 2 for this mixture experiment.

### RESULTS AND DISCUSSION

#### ORM Data Analysis

Glajch et al. (1) introduced a new method to evaluate peak separation for high performance liquid chromatography (HPLC) called overlapping resolution maps (ORM). A resolution map is generated for each pair of adjacent peaks. These maps are contours of the resolution measure for different mobile phases over the simplex mixture region. Any portion of the simplex mixture region that has resolution values greater than a desired minimum value represents a region of acceptable mobile phase solvents for that particular pair. A final mixture region is achieved that has the desired resolution for all component pairs by overlapping the acceptable regions for all adjacent pairs. This method can also be extended to situations where peak crossovers need to be considered.

The ORM method can be adapted to HPTLC by using the resolution function  $R_s(j, j+1)$  (6), which is defined in terms of the spot migration distance and spot width by:

$$R_s(j, j+1) = 2(D_{j+1} - D_j) / (W_{j+1} + W_j)$$

where  $D_j$  and  $D_{j+1}$  are migration distances of two adjacent spots;  $W_j$  and  $W_{j+1}$  are the spot diameters. Because we were only interested in separating adjacent spots, the problem of spot

crossover was not considered in this analysis. For the eleven dye compounds, ten resolution values were calculated for each mobile phase shown in Table 2. A Scheffe's second-order polynomial (9) was estimated for predicting the expected resolution of each pair of adjacent spots over the simplex mixture region.

The estimated Scheffe's polynomials can be used to generate contour plots for resolution values over the simplex mixture region by using PROC GCONTOUR in the computer package SAS (10,11). The ORM method requires generating ten contour plots and overlapping the acceptable regions. The acceptable region depends on the choice of an acceptable resolution value which is usually arbitrary. The problems of evaluating a number of plots and selecting an acceptable resolution value can be reduced by considering only the minimum resolution value at each mobile phase composition. The minimum resolution value represents the smallest separation of any two adjacent spots. If the minimum resolution values are contoured over the simplex mixture region, the largest separation of the two closest spots occurs in the contour region with the highest resolution values. Figure 1 shows the contours of the minimum resolution values for the eleven dye compounds. A SAS computer program in Table A.1 shows how to generate the contour plots of the minimum  $R_s$  values from the estimated second-order Scheffe's polynomials. These computer-generated contours are in transformed space and are redrawn for publication.

The best mobile phases are mixtures that are in the region bounded by the contour with a 0.30 value. The final blend was selected on the  $X_1$  and  $X_2$  boundary as 15% blend 1 and 85% blend 2 (i.e., 14.1% benzene, 63.8% chloroform, and 22.1% hexane). An additional experimental run at this final selected blend gave a value of 0.25 for the ORM method and a value of 0.52 for the IS method. Both values were considered to be within one standard deviation of the expected responses.

Using the minimum resolution contours requires only one plot and the acceptable mixture region is defined by the maximum contour value. There may be no feasible mixture region if the

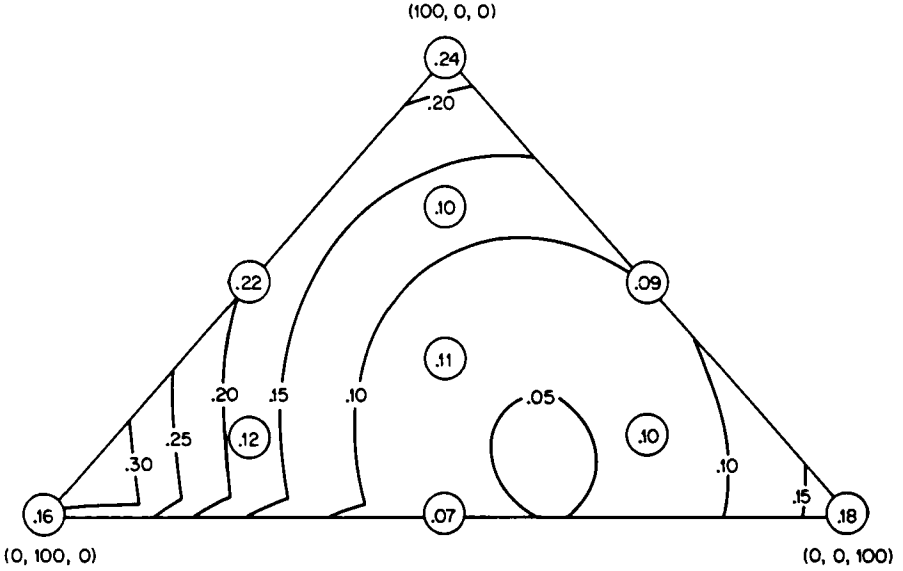


FIGURE 1. Contours of the Expected ORM Values with Experimental Values in the Circles. Coordinates of the Simplex Mixture Region are Volume Percents of the Three Binary Blends.

maximum contour of the minimum resolution values is less than a preset resolution criteria. This method shows that ORM is equivalent to the optimization method of maximizing over all experimental runs the minimum resolution function for each experimental run.

$$ORM = \text{MAX} [\text{MIN}(R_s(1,2), \dots, R_s(10,11))].$$

IS Data Analysis

A second optimization method called IS data analysis minimizes the ideal separation response function (3). This response function, originally identified as D6 by Bayne and Ma (3), is the sum of an ideal spacing term and an ideal spread term.



The response function is based on  $R_f$  values which are the fractions of the migration distances to the solvent front,  $L$  :

$$R_f = Z/L$$

where  $Z$  is the migration distance measured from the center of the spot. All experimental runs had a solvent front of  $L = 85$  mm and an elution time of 15 minutes.

The ideal separation of  $q$  components would have  $R_f$  values equally spaced on the unit interval  $[0,1]$ . The ideal value for the  $j$ -th ordered  $R_f$  value would be  $(j - 1)/(q - 1)$ . In addition to minimizing the distance between measured  $R_f$  values and ideal values, the response function also requires that the spots be spread out. This spread is measured by the standardized fourth central moment of the  $R_f$  values and is calculated by:

$$b_2 = M_4/M_2^2$$

where,

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

For  $q$  components with  $R_f$  values equally spaced in the unit interval, the exact value of  $b_2$  is:

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

The response function indicates how close the measured  $R_f$  values are to the ideal separation and ideal spread.

$$IS = \left\{ \sum [(R_f)_j - (j-1)/(q-1)]^2 + (b_2 - B_2)^2 \right\}^{1/2}.$$

The IS optimization procedure is to find mobile phases that minimizes the response function. The response function simultaneously optimizes the overall spot separation and is evaluated for each mobile phase in the simplex mixture region. A

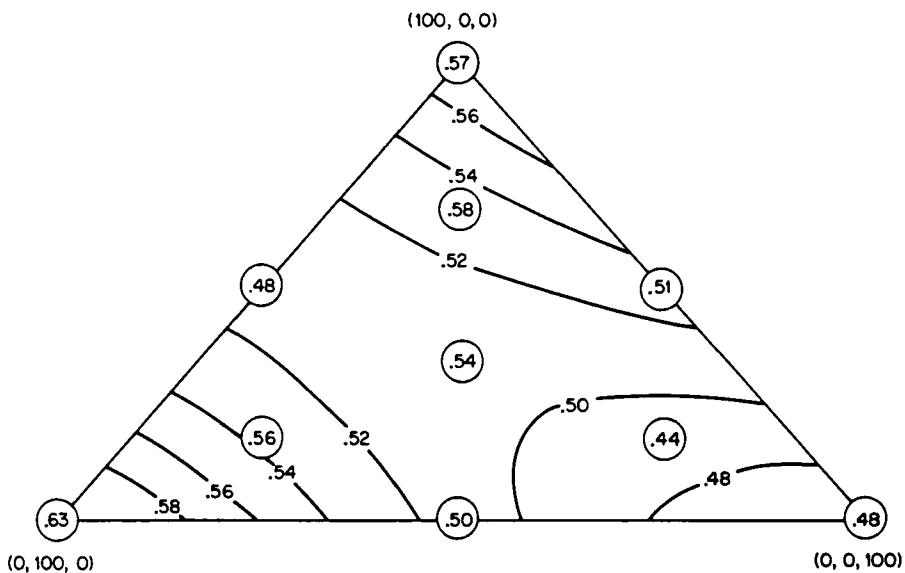


FIGURE 2. Contours of the Expected IS Values with Experimental Values in the Circles. Coordinates of the Simplex Mixture Region are Volume Percents of the Three Binary Blends.

second-order Scheffe's polynomial is estimated to approximate the expected IS response function over the simplex mixture region. Again, contours of the expected IS response can be plotted using PROC GCONTOUR and are redrawn in Figure 2.

The minimum ideal separation,  $IS = 0.46$ , occurs at 100% blend 3 (i.e., 53% THF, and 47% hexane). The measured IS values ranged from 0.46 to 0.63 and the standard deviation for an observed IS value is 0.05. These statistics indicate that the separations are fairly equal for all mobile phases in this study. Figure 3 shows the  $R_f$  values which are fairly uniform for all mixture compositions.

#### CONCLUSIONS

For HPTLC, we prefer optimization using the IS data analysis method rather than the ORM data analysis method. This preference

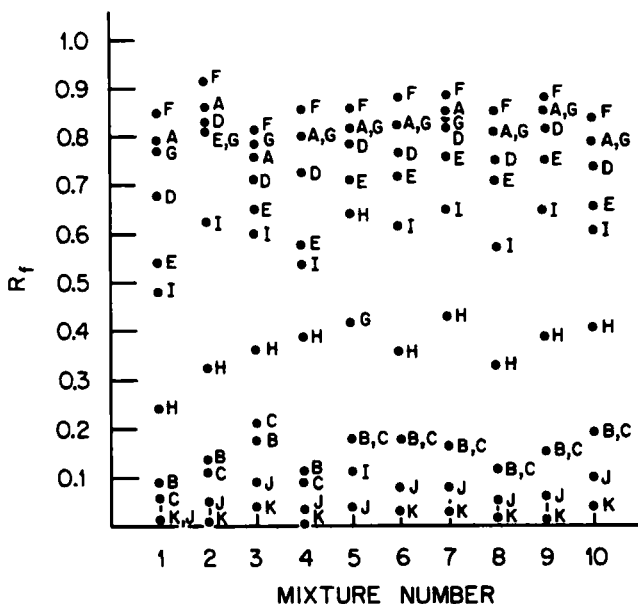


FIGURE 3.  $R_f$  Values for Each Mixture Number with the Compound ID Letter Indicating the Anthraquinone Dye.

is because additional HPTLC runs can easily be made that are specific to the worst case when nearest adjacent compounds are not separated.

The two data analysis methods result in different mobile phase compositions. The ORM method selected mobile phases that maximized the separation of the nearest pair of dye spots, while the IS method selected mobile phases that simultaneously maximized the overall separation of all dye spots. Selection of the data analysis method is therefore dependent on the specific objective of the optimization process.

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APPENDIX I

Table A.1. SAS Computer Program to Generate Response Contours.

```

*****
# GENERATE A DATA SET FOR CONTOUR PLOTS OF THE RESPONSE #
# MEASUREMENTS FROM SCHEFFE'S POLYNOMIALS #
*****;
DATA RESPONSE;
  DO V1 = -0.45 TO 0.85 BY 0.013;
  DO V2 = -0.75 TO 0.76 BY 0.015;
*****
# TRANSFORM AXIS FOR THE 2-DIMENSIONAL SIMPLEX MIXTURE REGION #
*****;
  X1 = (V1*SQRT(6) + 1.0)/3.0;
  X2 = (1.0 - X1 - V2*SQRT(2))/2.0;
  X3 = 1.0 - X1 - X2;
  X12 = X1*X2; X13 = X1*X3; X23 = X2*X3;
  RS1 = 0.00; RS2 = 0.00; RS3 = 0.00; RS4 = 0.00; RS5 = 0.00;
  RS6 = 0.00; RS7 = 0.00; RS8 = 0.00; RS9 = 0.00; RS10 = 0.00;
  IDEAL = 0.00; MINRS = 0.00;
  IF (0 < X1 < 1.0) AND (0 < X2 < 1.0) AND (0 < X3 < 1.0) THEN DO;
*****
# EVALUATE SCHEEFE'S PREDICTION EQUATIONS FOR ADJACENT SPOTS #
*****;
  RS1 = 0.28*X1+1.68*X2+1.67*X3+ 1.58*X12+3.82*X13+1.64*X23;
  RS2 = 0.82*X1+2.73*X2+2.53*X3+ 3.38*X12+6.11*X13-0.50*X23;
  RS3 = 1.07*X1+0.43*X2+0.73*X3+ 0.14*X12-2.79*X13-1.94*X23;
  RS4 = 3.59*X1+4.17*X2+4.22*X3+16.16*X12+5.96*X13+4.04*X23;
  RS5 = 4.13*X1+4.91*X2+4.05*X3- 1.19*X12+5.05*X13-1.29*X23;
  RS6 = 1.50*X1+3.23*X2+0.54*X3- 2.39*X12+5.64*X13-1.26*X23;
  RS7 = 2.49*X1+0.27*X2+1.41*X3+ 7.88*X12-5.93*X13+3.00*X23;
  RS8 = 1.38*X1+0.22*X2+0.89*X3+ 1.52*X12-1.33*X13-0.78*X23;
  RS9 = 0.23*X1+0.45*X2+0.19*X3- 0.56*X12-0.44*X13-1.01*X23;
  RS10 = 0.96*X1+0.61*X2+0.56*X3+ 0.01*X12+0.24*X13-0.03*X23;
*****
# CALCULATE THE IDEAL FUNCTION AND ORM FUNCTION #
*****;
  IDEAL = 0.58*X1+0.63*X2+0.46*X3-0.36*X12+0.05*X13-0.13*X2;
  MINRS = MIN(OF RS1-RS10);
END;
OUTPUT;
END; END;
*****
# CONTOUR PLOTS FOR THE RESPONSES #
*****;
GOPTIONS NOTEXT82;
PROC GCONTOUR DATA=RESPONSE;
  PLOT V1*V2=IDEAL/LEVELS = 0.0 0.46 0.48 0.50 0.52 0.54 0.56 0.58;
  PLOT V1*V2=MINRS/LEVELS = 0.0 0.05 0.10 0.15 0.20 0.25 0.30;
TITLE1 C=B 'CONTOURS';

```

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