8

Improved Resolution in High Performance Liquid Chromatography Analysis of Polynuclear Aromatic Hydrocarbons Using Ternary Solvent Systems

BARRY R. BELINKY

National Institute for Occupational Safety and Health,
4676 Columbia Parkway, Cincinnati, OH 45226

Polynuclear aromatic hydrocarbons (PAH's) are produced in most incomplete combustion processes. Examples are internal combustion engines, effluents from coal fired electricity generating plants, tobacco smoke, and from coking operations in steel and aluminum refineries. PAH's are also present in coal tar derived and coal tar containing products such as creosote and roofing pitch. They are found in the water we drink and the air we breathe, and are a ubiquitous component of our environment.

Concern for public health and safety is stimulated by the knowledge that many of these compounds are potent carcinogens. Past analytical efforts to relate chemical composition to carcinogenicity have used quantitation of the cyclohexane or benzene soluble fraction (BSF)(1) and of benzo(a)pyrene (BaP) (2,3,4,5) as indexes of carcinogenicity. However, the BSF is a nonspecific determination, as many nontoxic compounds are found in this fraction. BaP determinations can only give a crude estimate of carcinogenicity because (a) it is only one of many carcinogenic PAH's (b) the carcinogenic activity of different PAH's vary over a wide range and (c) the distribution of individual PAH's can fluctuate greatly from sample to sample. The vast complexity of PAH samples has been shown by Lao (6), who has identified over 120 PAH's in urban airborne particulate matter and Severson (7), who has identified about 900 PAH's in tobacco smoke. It is evident, then, that a need exists to more fully characterize the molecular species found in PAH mixtures.

Chromatographic Separation of PAH's

Both gas chromatography (GC) and high performance liquid chromatography (HPLC) have been extensively utilized in the separation of these complex mixtures. GC, especially when capillary columns are used, has been shown to have better overall resolving power than HPLC. HPLC coupled to a fluorescence detector, on the other hand, can provide sensitivities in the picogram range (8) as opposed to the nanogram range for GC/MS. In addition, stopped flow techniques allow UV or fluorescence spectra to be obtained for individual peaks, thus providing confirmation of structure. Improvement in resolving power of HPLC thus becomes an obvious goal in the effort to identify and quantitate mixtures of PAH's.

One way in which this can be accomplished is through the use of selective monitors. Selective UV detection has been demonstrated by, among others, Krstulovic and Brown (9) at the University of Rhode Island. Wheals (10) utilized selective fluorescence monitoring to differentiate peaks. Neither of these techniques actually improves resolution; they merely simplify the chromatogram either by eliminating peaks or by changing the ratios of overlapping peaks so that they may be determined mathematically. The drawback to such a procedure is that either multiple detectors must be used or multiple runs be made to obtain a complete analysis.

Another method is to optimize those chromatographic parameters which affect resolution. Although this paper is directed at a pragmatic approach to accomplishing this task, a brief detour into the mathematics is needed before we proceed. The fundamental equation describing chromatographic resolution, R_s is

$$R_s = \frac{1}{4}\left[\frac{\alpha}{\alpha - 1}\right]\sqrt{N}\left[\frac{k'}{1 + k'}\right] \qquad \text{Eq. 1}$$

where α, N and k' are, respectively, the separation factor, theoretical plate number and capacity factor.(24)

One can see three routes to an increase in the value of resolution. First, one can increase the plate number N which results in an increase in resolution proportional to the square root of N. The use of microparticulate packings was a major breakthrough in this respect. If we assume, however, that the chromatographer already has at his disposal a high efficiency column, then he must go to great lengths to obtain any significant increase in resolution because of the proportionality factor. Secondly, one can adjust k', the capacity factor. Figure 1 shows the relationship between k'/(1+k') and k'. Traditionally this is accomplished by adjusting the solvent composition so k' falls between 2 and 10, since resolution falls off rapidly below a k' value of 2, and relatively little increase is obtained for values of k' greater than 10. Finally the value of α, the selectivity factor can be adjusted. Obviously, one technique for changing the selectivity is to change the stationary phase. Indeed, HPLC analysis of PAH has been done on silica (11), alumina (12), cellulose acetate (13,14) and polyamide (13) columns and even silica columns with specialized bonded phases such as 3-(2,4,5,7-Tetranitrofluorenimine)propyldiethoxysiloxane (15). A change in mobile phase will also affect α. Therefore, in the present work, the ternary solvent system acetonitrile/methanol/water was examined over a wide range of concentrations to determine optimum mobile phase composition for PAH analysis. A statistical experimental design strategy was used for the optimization process.

Development of Optimal Mobile Phase

Most of the recent literature (16-20) describing PAH analysis by HPLC has centered on the use of octadecylsilane bonded stationary phases and either methanol-water or acetonitrile-water as the mobile phase. Both isocratic and gradient conditions have been utilized. The general approach taken to optimize chromatographic conditions usually is as follows. One of the two binary solvent systems methanol/water or acetonitrile/water is selected, and an arbitrary isocratic composition, say 60/40 or 70/30, is chosen. A standard mixture is chromatographed and then the organic/water ratio is adjusted to optimize k'. Depending upon the nature of the sample, a gradient may or may not be implemented to reduce analysis time while maintaining reasonable separation. If the resolution is insufficient for the analysis at hand, the other binary system is evaluated in the same manner. Figure 2 presents a triangular coordinate system, allowing visualization of all combinations of the three solvents. Vertex A represents 100% methanol, vertex B represents 100% acetonitrile, and vertex C, 100% water. Points X and Y represent the arbitrary binary solvent systems mentioned previously.

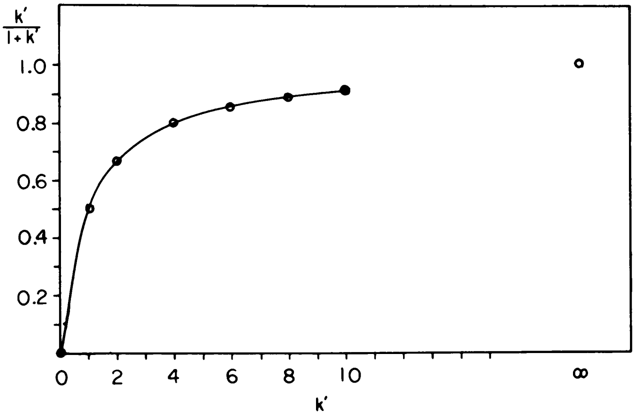

Figure 1. Plot of k'/(1 + k') *vs.* k'

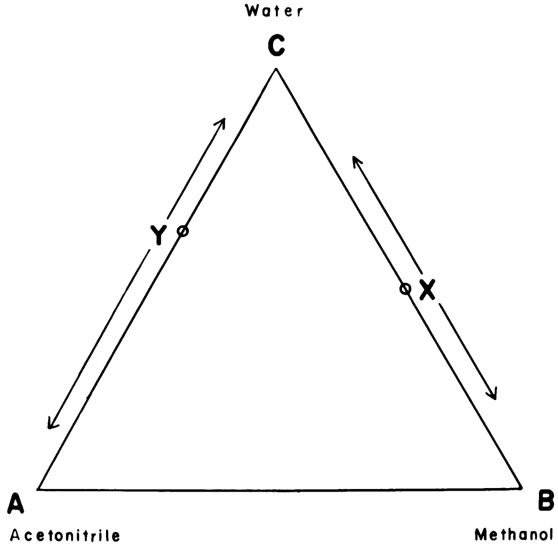

Figure 2. Representation of binary solvents X ***and*** Y ***as points in the ternary solvent system*** ABC

The arrows along the AC and BC axes are the ranges over which the solvent systems are varied.

Now the question arises: what happens in the center of this triangle where not two but three solvents are present? Are the capacity factors merely averaged? If so, α (which is the ratio of the capacity factors for solute 1 and solute 2) will not be enhanced. Or do more subtle effects occur? Karger (21), Snyder and Kirkland (22), Bakalyar (23) and others have discussed the expanded solubility parameter concept which says that polarity as defined by the Hildebrand solubility parameter is actually a composite of specific intermolecular interactions consisting of dispersive, dipole and hydrogen bonding interactions. No attempt will be made here to develop these subparameters on a theoretical basis. Suffice it to say that much is not yet understood regarding the quantification of these interactions -- especially in aqueous mixtures due to the unique properties of water. At any rate, we cannot yet predict values for resolution in a ternary solvent system based on theoretical considerations. To try to determine optimal solvent ratios in a ternary system by a trial and error approach would involve an inordinate number of experiments -- far more than most analysts would care to make. Nevertheless, a relatively simple way exists to experimentally optimize resolution in the ternary system.

Experimental

Fluoranthene (MC&B), Pyrene and Benzo(a)pyrene (Eastman Organic Chemicals), Benz(a)anthracene and Chrysene (Aldrich Chemical Co.) and Perylene (Pfaultz & Bauer) were used as received to make a standard solution with a concentration of approximately 0.1 mg/ml of each PAH.

Methanol and Acetonitrile (UV grade - Burdick & Jackson Laboratories) and water purified with a Millipore Super Q system were filtered through an 0.5 micron filter and degassed prior to use.

Instrumentation was a Waters Associates HPLC system including two Model 6000A pumps, a Model 440 UV detector fixed at 254 nm, a Model 660 solvent programmer and a Model U6K injector. A Vydac 201 TP reverse phase column (10 micron C-18 packing, 4.6 mm ID x 25 cm) was used throughout.

Discussion

The goal of this investigation was to determine optimum mobile phase composition for PAH analysis using the ternary solvent system acetonitrile/methanol/water. A model PAH standard solution was prepared consisting of three pairs of compounds listed in order of increasing retention: Fluoranthene-Pyrene, Benz(a)anthracene-Chrysene, and Perylene-Benzo(a)pyrene. The basic optimization strategy was as follows: 1) an isocratic ternary solvent system was found which gave maximum resolution of the fluoranthene-pyrene peaks at a k' = 5; 2) two additional isocratic ternary solvent systems were found to optimize the resolution of each of the other two PAH pairs; 3) a solvent gradient was constructed which incorporated all three isocratic compositions. All three optimal isocratic systems were determined simultaneously by means of a Simplex statistical design strategy.

The statisical design employed for the study requires that a bold approach be used: that is, both the upper and lower bounds of the region being investigated should be chosen such that they completely enclose the region of interest. Figure 3 shows this region. In the case of acetonitrile/methanol/water the two vertices where the eluent is strongest for the solutes of interest are pure methanol and pure acetonitrile. The weakest eluent is pure water. However, with pure water the PAH's would never be eluted from the column, so that particular choice is impractical. Therefore, a 65/35 methanol/water composition was chosen as the weakest mobile phase along the AC axis, and a 55/45 acetonitrile/water composition as the lower bound of the BC axis. These points are labeled C' and D' respectively. A Simplex design for a three component mixture requires a triangular coordinate system which obviously has only three vertices. Because of the constraint we have put on the upper bound for water content, not three but four vertices exist. By connecting points B and C', two roughly equal triangular areas are generated, each of which can be thought of as representing an independent ternary solvent system. These two triangular regions ABC' and BC'D' can be represented on an isometric orthogonal plot as seen in Figure 4. Vertex D' is technically a pseudocomponent rather than a component of the system because it is actually a mixture of the components B (acetonitrile) and C (water). Likewise, pseudocomponent C' is a methanol/water mixture. When reading the orthogonal graph, the line AB represents zero content of C'. Lines parallel to AB moving closer to vertex C' represent successively higher C' concentration. Acetonitrile is 100% at point B and decreases in a linear fashion to zero along the BA and BC' lines, and to 55% acetonitrile along the BD' line.

Our objective is to generate response surfaces which will allow the prediction of resolution at any point within the regions ABC' and BC'D'. The response surface is described mathematically by the special cubic model shown in equation 2.

$$Y = b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{123}X_1X_2X_3 \qquad \text{Eq. 2}$$

A measured value Y, which could be the capacity factor or resolution, is shown as the sum of the individual contributions of each component or pseudocomponent in the system. X_1, X_2 and X_3 represent the fraction of each pseudocomponent present. The b coefficients are determined experimentally. Note that there are no terms such as $b_{11}X_1X_1$ present because a solvent's interaction with itself has no physical meaning in such a system. The responses (Y values) are measured at each of the concentrations depicted graphically in Figure 5, which represents one of the two ternary systems previously described. The concentration of each of the pseudocomponents is given around the periphery. The seven closed circles are the experimentally determined responses at each vertex, at the midpoint of each binary system, and at the centroid of the triangle. These seven points will be used to generate the b coefficients in Equation 2. The three open circles are also experimentally determined values, and will be compared with values calculated from the special cubic model to determine the degree of fit. All ten values are determined in duplicate so that an estimate of precision can be made. The calculation of the "b" values is shown in Equations 3 through 9, Table I. Substituting the b values in Equation 2 will allow the prediction of Y for any given solvent composition within the confines of the model assuming the model is an accurate representation of the response surface.

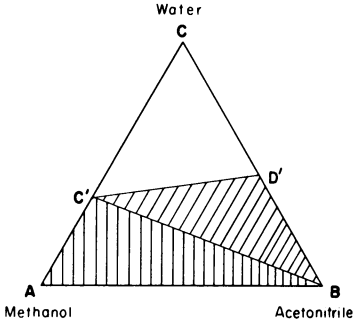

Figure 3. Ternary solvent systems ABC′ *and* BC′D′

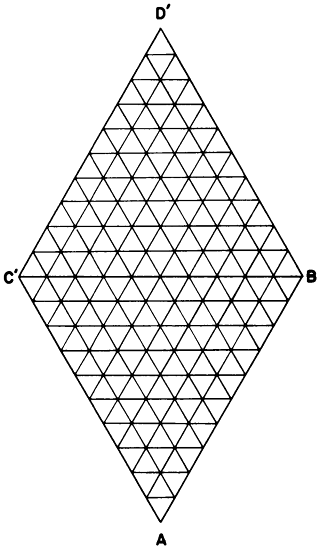

Figure 4. Orthogonal representation of ternary solvent systems ABC′ *and* BC′D′

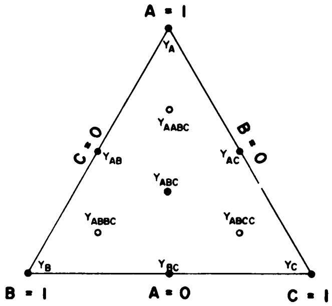

Figure 5. Ternary solvent system design; check points for determination of lack-of-fit indicated by open circles.

Table I

Calculation of Coefficients for Special Cubic Model

$$b_1 = Y_A \qquad \text{Eq. 3}$$

$$b_2 = Y_B \qquad \text{Eq. 4}$$

$$b_3 = Y_C \qquad \text{Eq. 5}$$

$$b_{12} = 4(Y_{AB}) - 2(Y_A + Y_B) \qquad \text{Eq. 6}$$

$$b_{13} = 4(Y_{AC}) - 2(Y_A + Y_C) \qquad \text{Eq. 7}$$

$$b_{23} = 4(Y_{BC}) - 2(Y_B + Y_C) \qquad \text{Eq. 8}$$

$$b_{123} = 27(Y_{ABC}) - 12(Y_{AB} + Y_{AC} + Y_{BC}) + 3(Y_A + Y_B + Y_C) \qquad \text{Eq. 9}$$

The three checkpoints Y_{AABC}, Y_{ABBC} and Y_{ABCC} in Figure 5 are used to verify the validity of the model. This is accomplished by examining the difference between the observed and predicted values of Y at each checkpoint. The lack of fit is given by the expression in Equation 10.

$$S^2_{LF} = \frac{1}{3}\sum_{i=1}^{3} (\bar{Y}_{oi} - \hat{Y}_i)^2 \qquad \text{Eq. 10}$$

where

$\bar{Y}_{oi}$ is the observed average response at th checkpoint, and $\hat{Y}_i$ is the predicted response at the ith checkpoint (from equation 2)

Duplicate values of Y for all ten points are used to determine the pooled error variance (Equation 11) and the replication error variance (Equation 12).

$$S^2_p = \frac{\sum_{j=1}^{10} (Y_{j1} - Y_{j2})^2}{20} \qquad \text{Eq. 11}$$

$$S^2_{\bar{Y}} = \frac{S^2_p}{2} \qquad \text{Eq. 12}$$

where

Y_{j1} and Y_{j2} are the duplicate response determinations at each of the ten solvent compositions.

And finally the lack of fit variance is compared to the replication error variance using an F-test (Eq. 13), which in this case being three components with ten degrees of freedom, is 3.71. If this ratio is less than 3.71, then the fit is good and the equation for the response surface can be considered valid.

$$\text{(F-test)} \quad S^2_{LF} \div S^2_{\bar{Y}} < 3.71 \qquad \text{Eq. 13}$$

A total of seventeen different mobile phase compositions are required to generate the response surface for the two Simplex designs as shown in Figure 6. Each run is done in duplicate, and the entire design is randomized to reduce experimental bias. Runs 12 through 17 are the checkpoints used to determine variance and goodness of fit. The overall design is shown in Table II.

Capacity factors, column efficiency in terms of N, and resolution were calculated for each of the six peaks in the chromatograms. Initial attempts to generate response surfaces using either k' or R_s were failures as indicated by the F-test in equation 13. New sets of coefficients for the special cubic model were generated, using log k' and log R_s as the measured responses, which gave acceptable F-tests. Orthogonal plots were then generated for each of the three pairs of test compounds. Although the response surfaces were generated using the log of the response, the isopleths, for clarity, are identified by the actual response, rather than the log of the response. Figure 7 shows the response surface for the capacity factor for fluoranthene as a function of solvent composition. The response surface for resolution of fluoranthene/pyrene is shown in Figure 8. Superimposed is the isopleth for a capacity factor of 5. The solvent composition corresponding to a k' of 5 at which resolution is greatest is shown by the circle. Response surfaces for the other two solute pairs were generated similarly. Figure 9 shows that resolution for benz(a)anthracene and chrysene is at a maximum within the lower triangular region. Again the point of maximum resolution for a capacity factor of 5 is indicated. The perylene/BaP response surface is shown in Figure 10. This Figure demonstrates the

Table II

Simplex Design for Ternary Solvent Optimization

Component					Composition (%)			Response
Run	A	B	C'	D'	Acetonitrile	Methanol	Water	Y
1	1	0	0	0	0	100	0	Y_A
2	0	1	0	0	100	0	0	Y_B
3	0	0	1	0	0	65	35	Y_C
4	0	0	0	1	55	0	45	Y_D
5	1/2	1/2	0	0	50	50	0	Y_{AB}
6	1/2	0	1/2	0	0	82.5	17.5	Y_{AC}
7	0	1/2	1/2	0	50	32.5	17.5	Y_{BC}
8	0	1/2	0	1/2	77.5	0	22.5	Y_{BD}
9	0	0	1/2	1/2	27.5	32.5	40	Y_{CD}
10	1/3	1/3	1/3	0	33.3	55	11.7	Y_{ABC}
11	0	1/3	1/3	1/3	51.7	21.7	26.7	Y_{BCD}
12	2/3	1/6	1/6	0	16.7	77.5	5.8	Y_{AABC}
13	1/6	2/3	1/6	0	66.7	27.5	5.8	Y_{ABBC}
14	1/6	1/6	2/3	0	16.7	60	23.3	Y_{ABCC}
15	0	2/3	1/6	1/6	75.8	10.8	13.3	Y_{BBCD}
16	0	1/6	2/3	1/6	25.8	43.3	30.8	Y_{BCCD}
17	0	1/6	1/6	2/3	53.3	10.8	35.8	Y_{BCDD}

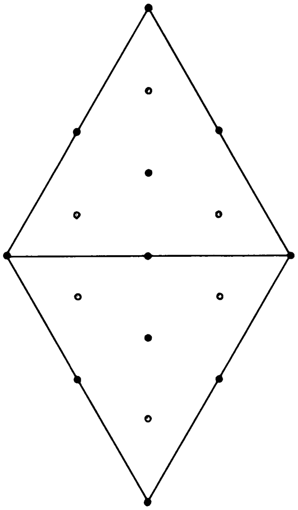

Figure 6. Schematic of two ternary solvent designs containing a common boundary

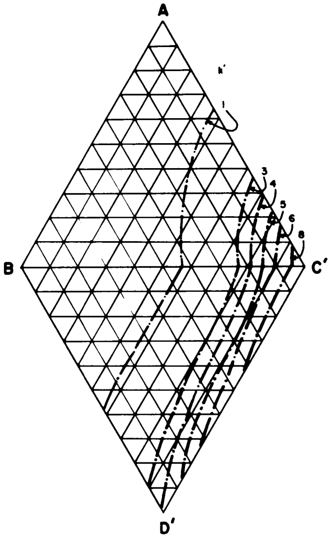

Figure 7. Response surface for capacity factor, k', *of fluoranthene*

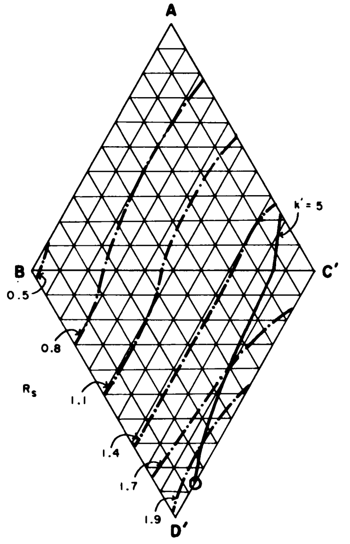

Figure 8. Response surface for resolution, R_s, of fluoranthene and pyrene; (——) is the isopleth for k' (fluoranthene) = 5.

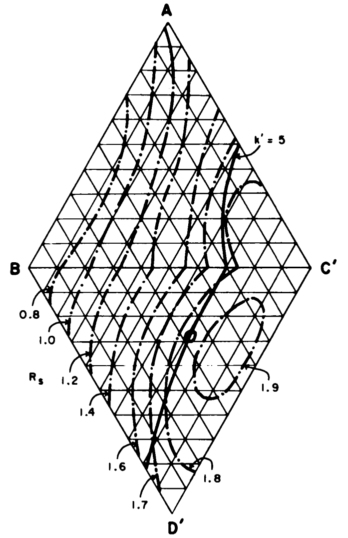

Figure 9. Response surface for resolution of benz(a)anthracene and pyrene; (——) is the isopleth for k' (benz(a)anthracene) = 5.

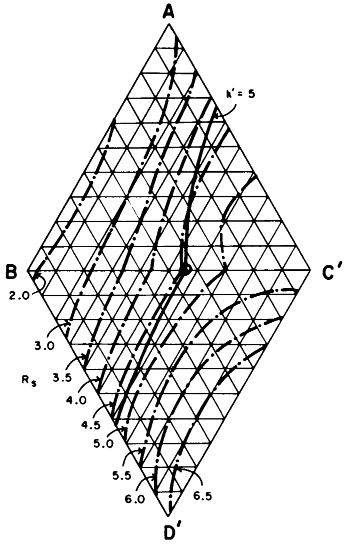

Figure 10. Response surface for resolution of perylene and benzo(a)pyrene; (——) is the isopleth for k′ (perylene) = 5.

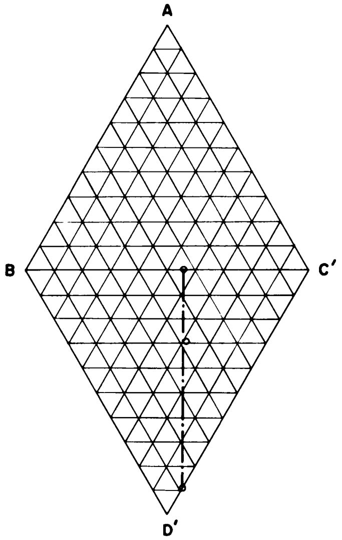

Figure 11. Optimized solvent gradient for acetonitrile/methanol/water ternary system

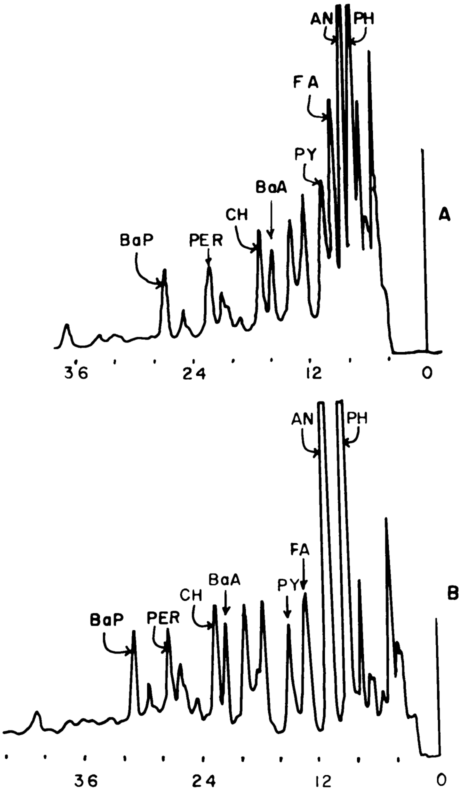

Figure 12. Comparison of binary and ternary solvent systems for "soft" coal tar pitch: (A) acetonitrile/water, 55:45 to acetonitrile/water, 72:28; linear, 30 min; (B) acetonitrile/methanol/water, 48:8:44 to acetonitrile/methanol/water, 43:37:20; linear, 30 min.

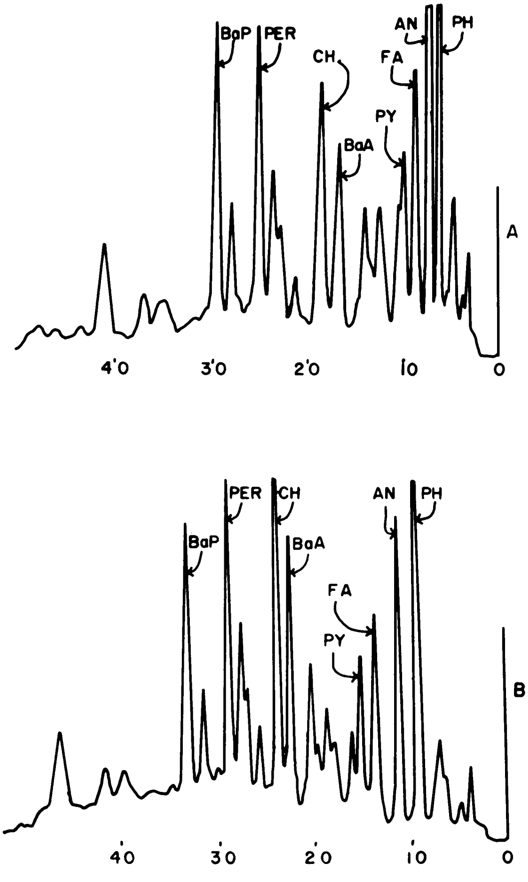

Figure 13. Comparison of binary and ternary solvent systems for "hard" coal tar pitch: same solvent ratios as Figure 13; 40-min linear gradient.

flexibility in choosing optimized solvent composition. The k' = 5 isopleth is roughly parallel to the R_s = 4.5 isopleth over the composition range indicated by coordinates A = 0.1, B = 0.39, C' = 0.51 to B = 0.62, D' = 0.38. In practice then, the choice of which optimal ternary solvent should be used may take other considerations (such as viscosity or ease of generating the final gradient) into account. In Figure 11 a gradient is drawn through the three points chosen as having maximum resolution at k' = 5 for each of the pairs studied. The initial mobile phase composition of this system is 48.4% acetonitrile, 7.8% methanol and 43.8% water. The final composition is 43% acetonitrile, 37% methanol and 20% water.

The resolution in actual samples for the optimized ternary mobile phases was compared to that obtained with the optimized binary mobile phase (acetonitrile/water) which had been used previously. Figure 12 shows a comparison of chromatograms obtained using the binary and ternary solvent systems. The sample was a soft coal tar pitch which contains a high proportion of low molecular weight PAH's. The six test compounds are identified in the figure, along with the two major components, phenanthrene and anthracene. Note the near baseline resolution of the fluoranthene/pyrene pair in the ternary system, and the resolution of the two large early-eluting compounds, phenanthrene and anthracene.

Figure 13 shows a similar pair of chromatograms of a binary sample of a hard coal tar pitch with many high moleular weight PAH's present. In the upper chromatogram, the pyrene peak has a shoulder, which is nearly completely resolved in the ternary system chromatogram. The two peaks between Pyrene and Benz(a)anthracene above are seen as four peaks in the lower chromatogram.

Summary

The use of an acetonitrile/methanol/water eluent has been shown to improve resolution of PAH chromatographed on a Vydac reverse phase column. This improved resolution should result in greater precision and accuracy in the quantitation of individual PAH. Determination of optimal solvent concentration is simplified through application of statistical design techniques. These techniques can be further utilized to investigate various ternary mobile phases in combination with different stationary phases.

Disclaimer

Acknowledgement

The author is indebted to Mr. Dennis Hill of the CDC Parklawn Computer Center who developed the programs to generate the response surface curves, and to Dr. Alexander W. Teass for valuable discussions.

Abstract

The analysis of polynuclear aromatic hydrocarbons (PAH's) presents one of the most challenging tasks to the occupational health chemist. The problems arise from the needs to separate large numbers of structurally similar compounds and simultaneously detect them at extremely low levels. High performance liquid chromatography (HPLC) is a frequently used tool for such analyses. This paper applies statistical experimental design techniques to the improvement of HPLC resolution through optimization of solvent selectivity effects in a ternary solvent system.

Literature Cited

1. Wallcave, L., Garcia, H., Feldman, R., Lijinsky, W., and Shubik, P. Toxicol. Appl. Pharmacol., (1971) 18, 41-52.

2. "Scientific and Technical Assessment Report on Particulate Polycyclic Organic Matter". U. S. Environmental Protection Agency (1975) No. PB 241 799.

3. Sawicki, E., Stanley, T. W., Elbert, W. C., Meeker, J. and McPherson, S. Atmos. Env., (1967) 1, 131.

4. Sawicki, E., Elbert, W., Stanley, T. W., Hauser, T. R. and Fox, F. T. Int. J. Air Poll., (1960) 2, 273.

5. Jackson, J. O., Warner, P.O. and Mooney, T. F. Jr. A. I. H. A. J., (174) 35, 276.

6. Lao, R. C., Thomas, R. S., Oja, H. and Dubois, L. Anal. Chem., (1973) 45, 908.

7. Severson, R. F., Snook, M. E., Akin, F. J., and Chortyk, O. T. In "Carcinogenesis, Vol. 3: Polynuclear Aromatic Hydrocarbons"; Jones, P. W. and Freudenthal, R. I., Ed.; Raven Press: New York, 1978.

8. Christenson, R. G. and May, W. E. J. Liq. Chrom. (1978) 1 (3)85.

9. Krstulovic, A. M., Rosie, D. M. and Brown, P. R. Anal. Chem., (1976) 48, 1383.

10. Wheals, B. B., Vaughan, C. G. and Whitehouse, M. J. J. Chromatogr., (1975) 106, 109.

11. Boden, H. J. Chr. Sci., (1976) 14 (8), 391.

12. Golden, C. and Sawicki, E. Anal. Let., (1976) 9 (10), 957.

13. Klimisch, H. J. J. Chromatogr. (1973) 83, 11.

14. Klimisch, H. J. Anal. Chem. (1973) 45 (11), 960.

15. Lochmuller, C. H. and Amoss, C. W. J. Chromatogr. (1975) 108, 85.

16. Eisenberg, W. C. J. Chr. Sci., (1975) 16 (4), 145.

17. Dong, M., Locke, D. C. and Ferrand, E. Anal. Chem., (1976 48 (2), 368.

18. May, W. E., Chesler, S. N., Cram, S. P., Gump, B. H., Hertz, H. S., Enagonio, D. P. and Dyszel, S. M. J. Chr. Sci., (1975) 13 (11), 535.

19. Oyler, A. R., Bodenner, D. L., Welch, K. J., Liukkonen, R. J., Carlson, R. M., Kopperman, H. L. and Caple, R. Anal. Chem., (1978) 50 (7), 837.

20. Fox, M. A. and Staley, S. W. Anal. Chem. (1976) 48 (7), 992.

21. Karger, B. L. J. Chromatogr. (1976) 125, 71.

22. Snyder, L. R. and Kirkland, J. J. "Introduction to Modern Liquid Chromatography" pp 215ff, John Wiley & Sons, Inc., New York (1974).

23. Bakalyar, S. R., McIlwrick, R. and Roggendorf, E. J. Chromatogr. (1977) 142, 353.

24. Snyder, L. R. and Kirkland, J. J. "Introduction to Modern Liquid Chromatography" pp 35-38, John Wiley & Sons, Inc., New York (1974).

25. "Strategy of Experimentation," Revised Edition, E. I. du Pont de Nemours & Co., Wilmington, DE, October, 1975, p. 51.

RECEIVED October 30, 1979.