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REVIEW

METHOD DEVELOPMENT IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING RETENTION MAPPING AND EXPERIMENTAL DESIGN TECHNIQUES

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1. INTRODUCTION

Systematic method development in high-performance liquid chromatography (HPLC) has been performed using different approaches, many of them detailed in accompanying papers in this issue. While each technique has certain advantages and disadvantages, difficult separations often require as much potential resolving power as possible to achieve a satisfactory final separation. In addition, it is desirable to have an efficient means of developing a separation in a reasonable time.

This review will concentrate on the retention mapping methods first described for reversed-phase HPLC in 1980[1] and subsequently broadened to include other separation modes and variables. The basic philosophy of retention mapping is to obtain enough data on a sample mixture in a limited set of experiments to entirely describe, or "map", the behavior of solutes under other conditions not explicitly tested (but still within the boundaries of the variables) during the initial experiments. In this way, it is possible to predict effectively how a desired separation can best be achieved,

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rather than relying on performing many separations and simply choosing the best one obtained.

In the general case, it is best to pick those variables or parameters of the separation, such as type of solvent, column, mobile phase composition and additives, that are most likely to affect (and hopefully improve) a particular separation. While the quantitative effect of different variables is often not predictable, liquid chromatographic theory has been developed to the state where the most important variables to effect a change in the separation can be selected as a function of the sample type and HPLC method chosen. For example, mobile phase composition is known to be very important in changing selectivity (band spacing) in reversed-phase HPLC. It is also known that changing from a methanol modifier to acetonitrile usually has a greater effect on the separation than changing to another alcohol, such as ethanol or isopropanol. Similarly, a change from a C_{18} to a cyanopropyl (CN) column is more likely to result in significant selectivity changes than changing from a C_{18} to a C_8 column[2].

Identifying the important separation variables is important, but another key to effective retention mapping is the choice of an appropriate set of experiments to determine the effect of these variables. Experimental design is well established in fields such as process control and optimization of product performance; however, the use of proper design strategies is relatively new to the field of analytical separations. The basic premise is to plan experiments to gather data that can then be used to predict results accurately under other conditions. In addition to being a more efficient method for gathering data, this approach allows easy automation of the entire method development process, as the general strategy is established before any experimental measurements are made.

One additional fact that must be considered is how to measure the quality of a separation in order to compare quantitatively the results of two experiments. Every optimization technique faces a similar dilemma: given two chromatograms, how does the user and/or automated system determine which separation is better? For a method development strategy to proceed in a controlled manner, this question must be addressed quantitatively.

The retention mapping and experimental design techniques that we have employed have been useful for various types of HPLC separations, and a brief review of each different method is supplied here.

2. MAPPING STRATEGIES FOR DIFFERENT MODES OF HPLC

2.1. Reversed-phase HPLC

The basis for organizing mobile phase selectivity in reversed-phase HPLC is the solvent selectivity triangle, first described by Snyder[3,4] and shown in Fig. 1. The premise of this approach is that solvent selectivity is governed by three main effects: the ability of the organic solvent to interact with the solute as a proton donor (basic), proton acceptor (acidic) or a dipole. In reversed-phase HPLC with water as the common diluent solvent, many potential HPLC solvents cannot be used, as they are not totally miscible with water. Considering miscibility, ease of use, availability, reasonable boiling point, etc., the best choices are methanol (proton acceptor), acetonitrile (proton donor) and tetrahydrofuran (dipole). The choice of these three

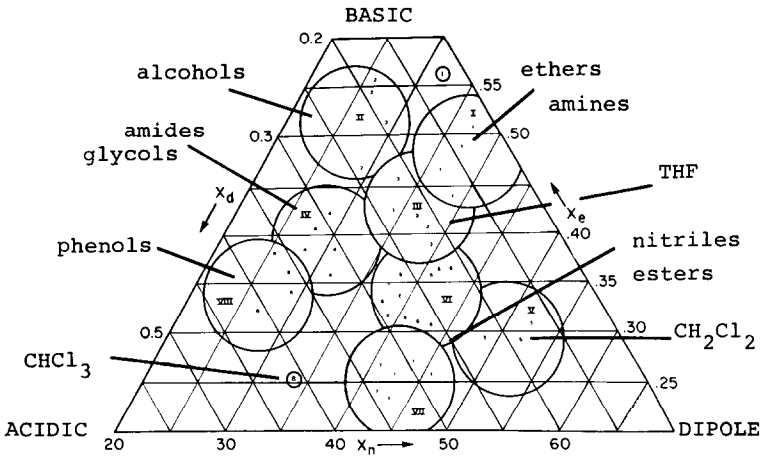

Fig. 1. Solvent selectivity triangle according to Snyder[3,4]. Reprinted with permission from ref. 4.

solvents to modify water for maintaining the proper eluting power (solvent strength) is only the first concern in proceeding with method optimization. The second choice involves which experiments to perform to determine efficiently the effects of these solvents on band spacing for the separation.

Each of these organic modifiers [with the correct amount of water to give similar retention(s) of the solutes] represents one "mixture variable" that can influence selectivity. As physical constraints require that the sum of all solvent modifiers total 100%, there are only two independent variables among the three mixture variables (the value of the third is dependent on the values of the sum of the other two). Therefore, appropriate experiments are based on a mixture design, shown in Fig. 2. Each corner of the triangle represents one binary mobile phase solvent [methanol–water at the top corner, acetonitrile–water and the lower left and tetrahydrofuran (THF)–water at the lower right].

Measuring solute retention in a sample in each of these three binary mobile phases shows band spacing changes as the type of modifier is varied (methanol to acetonitrile to THF). Often, a change of solvent type will result in significant relative retention changes. For example, the k' values of 2-methoxynaphthalene and naphthalene measured on a C_8 column[1] were both 4.0 in methanol–water (63:37); however, in tetrahydrofuran–water (39:61) the k' values were 4.6 and 5.2, respectively. The goal, however, is not simply to change the retention, but to find conditions where the separation of all solutes of interest is best. If retention was a simple linear function of solvent type, the three binary mobile phase experiments (methanol–water, acetonitrile–water and THF–water) would be sufficient to define retention in any combination of the solvents. However, it has been found experimentally that this is not the case; rather, a higher order dependence of retention on solvent composition is typical. In some instances, this behavior can be complex; fortunately for most reversed-phase systems, it can be approximated by a second-order polynomial fit to the retention data. In this case, retention is described as

$$k' = A\varphi_1^2 + B\varphi_2^2 + C\varphi_3^2 + D\varphi_1\varphi_2 + E\varphi_1\varphi_3 + F\varphi_2\varphi_3 \quad (1)$$

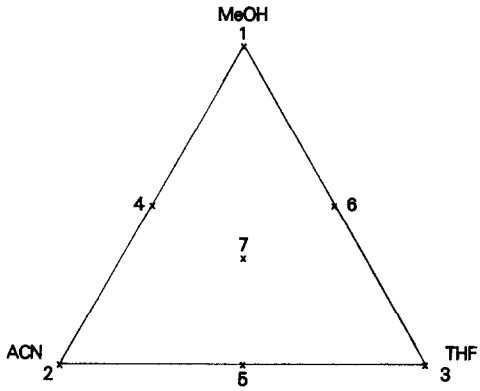

Fig. 2. Mixture-design experiments used for evaluating solvent selectivity. MeOH = Methanol, ACN = acetonitrile, THF = tetrahydrofuran.

where k' is the capacity factor of a peak of interest, φ_1, φ_2 and φ_3 are the relative concentrations of the mixture variables ($\varphi_1 + \varphi_2 + \varphi_3 = 100\%$) and A–F are constants for any one particular solute. As eqn. 1 involves six unknown variables (A–F), six measurements at different values of φ_1, φ_2 and φ_3 are required to solve a series of simultaneous equations for the values of the constants. Once these solutions have been obtained, it is possible to predict the k' for that solute under any other conditions of φ_1, φ_2 and φ_3, *i.e.*, any other solvent composition.

Although only six different solvent compositions are required, in practice a seventh experiment is added to accommodate experimental uncertainty and lack of fit to the presumed second-order equation. The result is the mixture-design experimental setup shown in Fig. 2. In theory, any seven points within this triangle can be chosen, but in practice, if the points are chosen as shown, are evenly separated and as far apart from each other as possible, the fit to the second-order equation is more accurate. Data obtained for 2-methoxynaphthalene in the previously described example on a C_8 column using methanol, acetonitrile, THF and water was fitted to the second-order equation and the coefficients were determined. The resulting retention map plotted as actual retention times is shown in Fig. 3. This map predicts the retention for this solute anywhere within the solvent composition triangle, based only on the results of the measured seven points. How to use this information on each peak to predict the best conditions for separation of all peaks is described in a later section.

2.2. *Normal-phase HPLC*

The solvent selectivity effects in normal-phase HPLC are based on a similar solvent triangle to that shown in Fig. 1. The effects for normal-phase operation are described based on the ability of a solvent to exhibit localization effects with respect to the stationary phase. The major solvent types are those able to act as non-localizing, basic localizing or non-basic localizing solvents; this effect was described in further detail elsewhere[5]. The result is that a wider range of potential solvent effects is available, as the diluent is a non-polar organic, such as hexane or (preferably) 1,1,2-trifluoro-1,2,2 trichloroethane (FC-113)[6]. In particular, the organic solvents used are more miscible with the non-polar diluent solvent. A useful choice of selectivity solvents is methylene chloride as a non-localizing solvent, methyl *tert.*-butyl ether

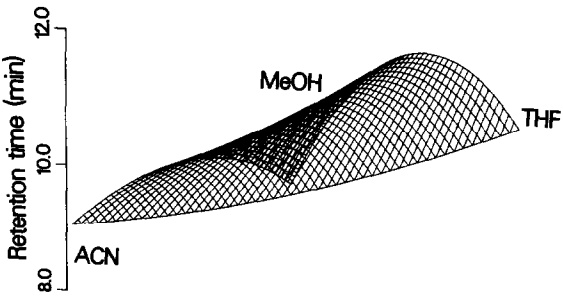

Fig. 3. Retention time map for 2-methoxynaphthalene as a function of solvent composition. Data from ref. 1 using the experimental design approach of Fig. 2.

(MTBE) as a basic localizing solvent and ethyl acetate or acetonitrile as a non-basic, localizing solvent. Methanol may also be used as a proton acceptor and will occasionally result in different selectivity effects. FC-113 is the preferred diluent, as it is completely miscible with all of these modifiers in all propertions; hexane and heptane have limited miscibility with methanol and acetonitrile.

Original work in normal-phase optimization was limited to unmodified silica columns; however, more recent work has utilized polar bonded phases such as amino and cyano columns. De Smet *et al.*[7] used a CN column for method development in pharmaceutical analysis. The CN column is particularly useful, as it can be used in both normal- and reversed-phase modes, and is often the column of choice for a first separation in some laboratories when both types of HPLC are being considered.

Although most practical separations are performed in the reversed-phase mode, optimized normal-phase separations are a powerful alternative. As the selectivity triangle approach described above is used to examine the effects in normal-phase HPLC, the same mapping and optimization strategies used for reversed-phase HPLC can be employed to develop a method and find a final separation. Relative retention shifts (α) of two-fold or more have been observed for solutes in different mobile phase during solvent selectivity scouting[8]. In fact, the potential for greatly changing (and improving) the separation in normal-phase operation by changing the solvent type is usually greater than in the reversed-phase mode. In addition, the normal-phase mode is often used for preparative separations in which the column is often overloaded with sample. The ability to optimize the separation of a solute of interest from other components in the sample is particularly important. Finally, normal-phase operation is often the method of choice for the separation of isomers.

2.3. Ion-pair HPLC

Ion-pair HPLC is a useful alternative to reversed-phase HPLC, especially when the mobile phase components are basic or acidic. This technique uses an aqueous–organic mobile phase that contains a buffer to control pH and an ion-pair reagent to provide more retention and selectivity than is available from a simple aqueous–organic system. Varying the organic modifiers such as from aqueous methanol to aqueous acetonitrile sometimes does change the selectivity just as in reversed-phase systems. However, a more general and powerful approach to changing band spacing is to alter the pH and the concentration of the ion-pair reagent in the mobile phase.

The choice of ion-pair reagent is often considered important to attain the proper retention for separation. While different reagents, such as hexylsulfonate *vs.* octylsulfonate, do affect retention and selectivity, the same effects can be achieved by varying the concentration of one chosen reagent[9,10]. Therefore, it is convenient to use a single ion-pair reagent such as hexylsulfonate and vary the band spacing and selectivity by changing the concentration of this reagent.

A useful strategy for method development in ion-pair HPLC is shown schematically in Fig. 4[11]. The seven-experiment design resembles that used for both reversed- and normal-phase separations, but the new "diluent" solvent is now methanol, which is used primarily to adjust the retention of all compounds to an optimum k' region. Band spacing is affected by using (1) pH 2.5 buffer with no ion-pair reagent, (2) pH 7.5 buffer with no ion-pair reagent and (3) a pH 5.5 buffer with a maximum ion-pair concentration (typically 200 m*M*). Intermediate mobile phases 4–7 are obtained by mixing the three "corner" mobile phases for intermediate selectivity.

Retention mapping and optimization then proceed as in the reversed-phase system described above to determine selectivity effects on the individual solutes and find the best mobile phase region for separation. One complication sometimes does arise in ion-pair chromatography, namely that the change in retention is not as regular as observed in reversed-phase HPLC. It is often difficult to fit retention data well to a second-order polynomial equation (eqn. 1), and additional data points are needed near the corners of the triangle. This effect does not negate the use of this approach for probing selectivity changes and routinely developing methods; however, a few extra runs are sometimes needed to finish the optimization. Lack-of-fit is usually apparent when a predicted optimum and the actual separation differ substantially, and this is a clue that additional runs may be needed for an accurate prediction of the optimum mobile phase.

2.4. Stationary phase selectivity

Solvent selectivity is usually the most powerful method for improving a separation in HPLC. However, occasionally there are reasons to consider column bonded-

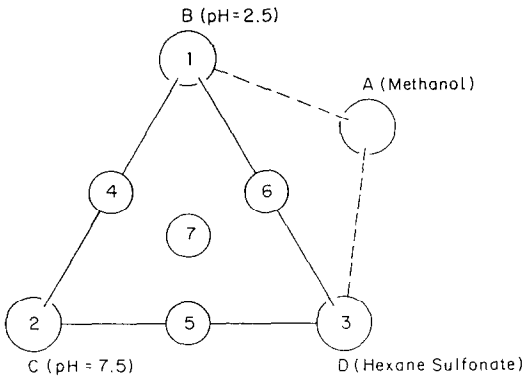

Fig. 4. Experimental design approach used for ion-pair chromatography. Reprinted with permission from ref. 11.

phase selectivity, such as changing from a C_8 bonded phase to a CN bonded phase in reversed-phase separations. One example is if it is inconvenient to change or mix particular mobile phase modifiers, or if a sample is incompatible with certain solvents. In such instances (and to offer an additional selectivity beyond that achievable by varying the mobile phase), changing to a column with a different stationary phase can be considered. In reversed-phase HPLC, there are a wide range of column types available, but three general types seem to offer the most opportunity to change band spacing[2]. An *n*-alkyl bonded phase, such as C_8 or C_{18}, is usually the first choice. A second type is a CN bonded phase, owing to its more weakly retentive nature, and a third alternative is a phase based on an aromatic silane, such as benzyl or phenyl.

Although not generally recommended for the best overall effect, changing the stationary phase in the column to produce selectivity changes does have some distinct advantages. One advantage is that mixed column systems (either serially connected or mixed-bed columns) exhibit solute retentions in direct proportion to the linear combination of the different stationary phases. Retention of a particular solute in an aqueous–organic mobile phase in a mixed stationary phase system can be described by

$$k'_{\text{Mix}} = \varphi_1 k'_{C_8} + \varphi_2 k'_{\text{CN}} \tag{2}$$

where φ_1 is the fraction of C_8 bonded phase and φ_2 is the fraction of CN bonded phase in the mixed bed or serial column system. For example, if a sample has a k' of 6.0 on a C_8 column and 4.0 on a CN column, then k' will be 5.0 on a column of containing equal amounts of C_8 and CN phases. As this equation is linear with column proportions, retention measurements can be made on all three individual column types (C_8, CN and phenyl, for example) and interpolated directly to any mixed column system containing any proportions of those three bonded phases.

A major disadvantage of using mixed stationary phases is that only certain combinations are readily available in many laboratories. In one study, both column and mobile phase selectivity were examined for a sample containing twenty phenylthiohydantoin (PTH)-amino acids[12]. Six variables (three organic modifiers and three different column types) were examined, and it was determined that only four of these variables were needed to obtain essentially all of the useful selectivity.Therefore, a convenient choice was to use one column and three organic modifiers rather than to mix columns of different stationary phase types for different separations. Therefore, in most instances, changing the band spacing selectivity by using mixed stationary phases is not as appropriate (or as powerful) as using mixed-solvent mobile phases.

2.5. Gradient elution

Much of the retention mapping and optimization in HPLC has been performed with isocratic mobile phases, but the process can be easily extended to include gradient elution separations. This separation mode is generally required when a sample contains solutes of widely differing retention. In considering gradient elution, the solvent selctivity triangle must be expanded to a fourth dimension to include explicitly the base solvent (for example, water in a reversed-phase system). This fourth variable has been shown diagrammatically as either a prism[13] or a tetrahedron[14]; although the latter description is mathematically and physicallv more correct, we have chosen to use a prism for better clarity, as illustrated in Fig. 5[13]. Here, methanol, acetonitrile and

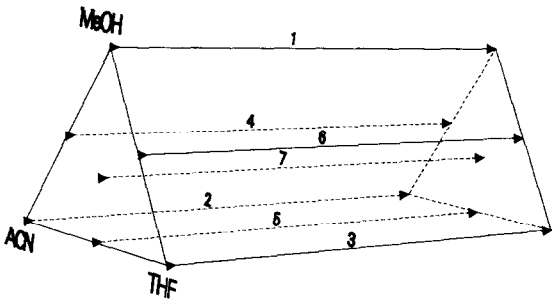

Fig. 5. Experimental design approach for gradient elution separations. The length of the prism corresponds to different proportions of water in the organic solvents. Reprinted with permission from ref. 13.

tetrahydrofuran are the organic modifiers, water is the base solvent and the plane containing the seven points represents a set of isoelutropic mobile phases (constant solvent strength) used for an isocratic optimization.

Gradient elution occurs when the composition of the mobile phase is varied from a weak solvent to a stronger solvent during the separation. An example is illustrated by arrow 1 in Fig. 5 for a simple methanol–water gradient, shown as going along one edge of the solvent tetrahedron. The rate of change of solvent strength can by itself lead to selectivity changes. This effect is examined in another paper in this issue[15]. The approach described in Fig. 5 relates to changing selectivity in gradient elution by changing the solvent organic modifiers, such as methanol instead of acetonitrile or THF, analogous to the isocratic method development described previously.

The experimental design for method development by gradient elution is similar to that used for isocratic separations, except that instead of maintaining solvent strength constant (isocratic), the rate of change of solvent strength is kept constant from run to run. The seven runs used in the optimization process are shown diagrammatically in Fig. 5. These exactly correspond to the seven mobile phases used for optimizing an isocratic separation (see Fig. 2). Retention and selectivity changes from run to run can be assessed in the same way as for the isocratic method. However, as the peak widths of different solutes are usually constant in gradient elution, the measure of separation between peaks can be taken simply as the difference in retention times. This is unlike an isocratic system, where the resolution between peaks is a function of both the retention and peak width of adjacent peaks.

In addition to this typical gradient elution scheme, more complicated (and potentially more powerful) systems can be devised where the solvent strength and solvent type selectivity can be simultaneously varied during the run[13,17]. These other approaches have found little practical use to date, but could be important for very difficult separations that cannot be optimized by the simple techniques.

3. CRITERIA FOR SEPARATION AND OPTIMIZATION

An appropriate strategy for accurately and efficiently mapping retention as a function of significant variables is only the first step in developing an optimum method. Once data for retention or resolution maps have been obtained, the operator must decide how best to measure separation quality so that the best or "optimum"

separation can be determined. There are many ways to describe the quality of a separation, including separation resolution between all adjacent peaks, total time of analysis and various functions that attempt to place a single value on the quality of a particular needed separation. This is a critical aspect of any optimization scheme, as the quantitative comparison of two or more chromatograms determines which is "better" and by how much. This subject is far too detailed for a complete analysis in this paper, but has been addressed by Schoenmakers[18].

Our usual choice of quantitative measurement is the overlapping resolution mapping (ORM) method. This is an expansion of earlier window-diagram approaches, first proposed by Laub and Purnell[19] for gas chromatographic separations. The basic approach is shown in Figs. 6 and 7 (date from ref. 1). The plots in Fig. 6 are retention maps for the solutes naphthalene and 2-methoxynaphthalene as a function of mobile phase composition within the solvent triangle space experimentally examined. When these two plots are intersected (or overlapped), the relative separation of these two peaks is obtained, as shown in Fig. 7. For the case of a gradient elution separation, this plot will be a simple retention time difference (Δt_R); for an isocratic separation, the peak width must also be included in this mathematical function in order to plot resolution, R_s, as

$$R_s = (t_2 - t_1)/[(w_1 + w_2)/2] \tag{3}$$

where t_1 and t_2 are the retention times of the two peaks and w_1 and w_2 are their respective baseline peak widths. Exact values (measured) of the two peak-width values

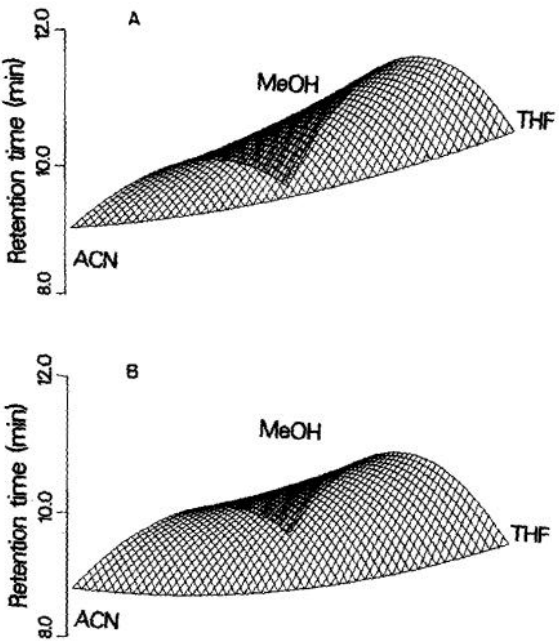

Fig. 6. Retention time maps for (A) 2-methoxynaphthalene and (B) naphthalene as a function of solvent composition. Data from ref. 1 using experimental design approach of Fig. 2.

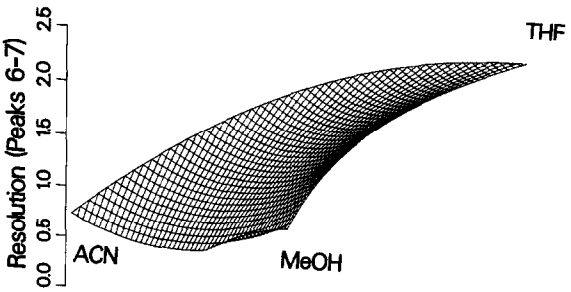

Fig. 7. Resolution map for peaks 6 and 7 (2-methoxynaphthalene and naphthalene) as a function of solvent composition. Data from ref. 1 using experimental design approach of Fig. 2.

can be used for the calculation. However, often the plate number in a separation does not significantly vary, and the width for each peak can be approximated by the expression

$$w_i = 4t_i/\sqrt{N} \tag{4}$$

where N is the column plate number and t_i is the retention time of the ith peak. For most practical cases, the difference between the W_i calculated in this way and an exact, measured peak width will result in a very small difference in the R_s value.

Once the resolution maps (see Fig. 7) have been determined for every peak pair in the chromatogram, these maps are overlapped (using a computer program). At each point within the solvent-triangle space, the limiting (smallest) resolution of any peak pair of interest within the set can then be determined. As a practical matter, the solvent triangle space is usually broken up into equal regions, and this process is evaluated for each region (usually 2500 points). The resulting overlapping resolution map in Fig. 8 with the substituted naphthalene data shows that for all nine solutes (36 total peak pairs), the best area for separation occurs in a ternary solvent mixture containing acetonitrile–THF–water, with a resolution of 2.5.

This method of peak-resolution measurement and optimization concentrates on the minimum resolution of the worst-resolved peak peak pair in a chromatogram as a measure of the quality of the chromatogram. The example shown above assumes that all pairs of peaks are important and must be resolved to the same degree. The ORM technique also applies to systems where only a subset of peak pairs is of importance, for example in Fig. 8 where only the naphthalene peak needs to be separated from all others, but the other peaks do not need to be resolved from one another. In this instance, only the peak pairs of interest are overlapped in the analysis; the others are ignored as they are unimportant to the final result.

The ORM technique does not explicitly use peak analysis time as a criterion for quality of separation. The time factor is handled in two ways. First, the overall strategy of experimental design for the various isocratic and gradient elution techniques assumes that mobile phase systems are chosen with approximately constant solvent strength (or change in solvent strength for gradient elution). This means that the analysis time for different runs should be approximately the same (within *ca.* 30%) for various conditions. In addition, once band spacings have been optimized using the

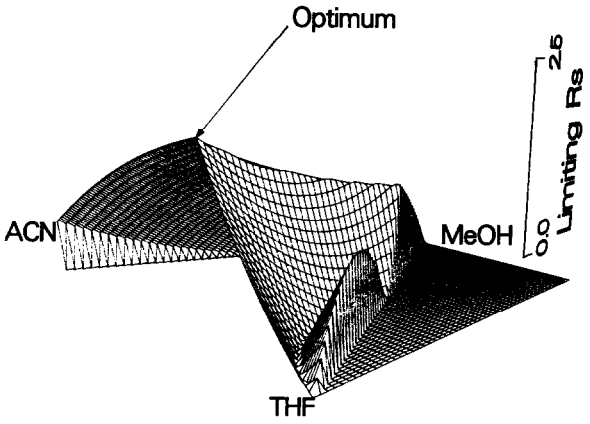

Fig. 8. Overlapping resolution map (ORM) for all nine substituted naphthalenes as a function of solvent composition. Data from ref. 1. Reprinted with permission from ref. 20.

techniques described above, further time savings are achieved using DryLab software column condition optimization methods for flow-rate, particle size, column length, etc.[21,22]. As chemical selectivity is defined, the physical parameters addressed with DryLab are independent of band spacing optimization.

4. PEAK TRACKING, SOFTWARE AND RUGGED SEPARATIONS

The use of retention mapping and experimental design techniques for method development and optimization can be powerful, but there are a few problems with their routine, practical implementation in many laboratories. The major limitation has been the need to follow accurately or "track" the peaks as parameters such as mobile phase composition, pH, or column type are changed. This problem is particularly crucial as those changes which cause the greatest peak movement are often those with the greatest potential for improving the separation (by definition, if the peaks never move, they are easy to track, but the separation cannot be improved!). While this is a difficult problem conceptually, a number of methods have been proposed which have minimized the problem, even if it has not been eliminated.

The first method is to inject standards for all known solutes in the system under all conditions. This is time consuming, but this time can be reduced by mixing a number of standards within each mixture and following these subsets. For example, if twelve solutes are being analyzed, rather than one injection of all twelve, or twelve injections, each with one solute, a series of four subsets can be prepared. Each subset would have four of the compounds of interest preferably with compounds having large differences in retention. As a cross-check, there should be crossover between subsets from one injection to another. A related method involves preparing different dilutions of each solute in the same mixture, using relatively large differences in peak area or height to aid in identifying and tracking peaks.

These methods are not useful for samples where the identities of some or all of the compounds are unknown, or where standards are not readily available, such as degradation products or impurities. In these instances, multiple wavelength detection,

such as with a diode-array UV detector, or more specific detection methods such as on-line liquid chromatography–mass spectrometry, can be useful. However, these techniques can also be ambiguous, and comprehensive, reliable peak tracking is still needed for many of these mapping strategies for successful optimization.

Another aspect of the retention mapping optimization that has been a concern during the past decade is the lack of easily available software to perform some of the data analysis and prediction functions. Although the initial paper on the retention mapping and ORM technique described well established mathematical functions and their application to this field, the initial commercialization of systems such as Sentinel precluded widespread dissemination of the software for routine use of the ORM method with other HPLC systems. However, a recent publication of the software code[23] and successful implementations of the theory by others[24–26] have minimized this problem. In addition, many users have discovered that even without the quantitative aspects of the ORM software, using the exprimental design strategy often led to a satisfactory separation in a reasonable time by manually examining the results of the seven prescribed runs.

Another benefit of the retention mapping techniques is that additional information exists that is not typically available using other methods for optimizing a separation. In particular, since the retention maps are interpolated from the prescribed experiments, data exist as to how "rugged" the final separation will be with respect to changes in any other the examined variables. For example, if the final separation is predicted for a mobile phase composition region in which selectivity does not change significantly with a 1% volume change in a solvent, this solvent region obviously would be more desirable than conditions where a slight change would cause the loss of a critical resolution. Also, if the separation changes slightly with time, owing to column aging or minor changes in solvent characteristics, the retention mapping data can often be used to determine which variable to change in order to obtain the desired separation without redoing method development.

5. CONCLUSIONS

Retention mapping using a proven experimental design strategy can be a powerful technique for method development and optimization in HPLC. This approach relies on a basic understanding of the parameters that are most likely to affect and improve a separation as a function of chemical selectivity. The technique has proved useful for reversed-phase, normal-phase, ion-pair and gradient elution HPLC and also column selectivity optimization, and combinations of these forms. Retention mapping assumes a reasonable adherence of retention to an assumed mathematical form (such as a second-order equation for k') as a function of most variables. The ability to automate much of the procedure and measurements permits efficient scouting of the most important variables affecting the separation. The use of computerized calculations is a helpful, but not necessary, tool in determining optimized conditions for the final separation. In addition, chemical selctivity mapping can be combined with methods such as DryLab for column conditions optimization and solvent strength optimization procedures described elsewhere in this issue[15]. Finally, knowledge gained in developing the retention maps for a final separation can be extremely useful in fine-tuning the separation to be more rugged, and in predicting conditions to change when a slight loss of resolution is observed with time.

6. SUMMARY

Method development in high-performance liquid chromatography (HPLC) using retention mapping and experimental design techniques is reviewed. The general strategy of overlapping resolution mapping is overviewed. A summary of various applications is examined for reversed-phase, normal-phase, ion-pair, and gradient elution HPLC, as well as stationary phase selectivity. In addition, numerical criteria for separation and optimization are detailed and a discussion of peak tracking and software is included.

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