Establishing a Selectivity Survey as Part of the Method Development Activity for an Isocratic Reversed-Phase Liquid Chromatographic Method

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Abstract

Often high-performance liquid chromatography method development is done by choosing a single C18 column and optimizing only the mobile phase composition. In this paper, it is demonstrated how to evaluate and optimize the best combination of the different stationary phase chemistries and mobile phases for a limited method development activity. By using column and mobile phase switching, it is possible to automate most of the activity in a nine-step process. Columns are chosen to represent the range of selectivity currently available. Interestingly, although the most popular column is the C18 phase, it is not the best column for the optimized methods in the cases studied.

Introduction

Developing a truly optimized high-performance liquid chromatography (HPLC) method requires the investigation of a large number of chromatographic variables. Such variables as buffer type and concentration, pH, ionic strength, additive type (if used), mobile phase composition, etc. all have to be manipulated. And, for individuals desiring this completeness, a computer-assisted approach is highly recommended (1). There are presently commercially available software packages for doing the complete optimization. However, for many other investigators, choosing a few variables to be fixed and attempting to "optimize" around only a limited number of parameters can make sense for finding the "best case" option(s) for the separation that meets the needs of the analyst.

In a typical limited HPLC method development scheme, the separation is achieved on an alkyl column, usually a C8 or C18 phase, using a mobile phase blend of organic and aqueous solvents. If the initial separation attempt on the alkyl column is not successful, the mobile phase is changed to another organic solvent. Once a separation is obtained, it is often used without any further activity. However, occasionally, after an initial separation is obtained, the same conditions are used on a group of alkyl columns from different manufacturers to investigate whether any improvements over the initial separation can be achieved. This approach focuses primarily on achieving the separation using the differences in mobile phase selectivity (2) with secondary selectivity enhancement caused by the various bonded phases. These additional selectivity changes achieved on different columns result from differences in the surface coverage of the alkyl phases and silanol content (e.g., endcapped versus nonendcapped phases) on the silica surface (3).

Larger selectivity variations could be offered by including different types of bonded phases in the scheme. For instance, it has been shown that a C8 or C18 phase used in conjunction with phenyl and cyano (CN) columns shows a much broader range of stationary phase selectivity (4). Thus, for some separations, using a CN or phenyl phase may be the most appropriate column to use. This difference in column phase chemistry can be a very powerful tool when performing method development and is often overlooked in favor of the popular C18 or C8 phase and mobile phase manipulation. Therefore, by combining column selectivity differences with variations in solvent strength and composition, an additional perspective in obtaining an "improved" separation is achieved. Investigating different bonded phases broadens the typical separation development approach of using a single type of stationary phase (5–8).

Recent reports (9,10) have demonstrated that with suitable column switching valves (CSV), solvent selection valves (SSV), and software to control these valves, it is possible to automate large portions of a method development process. Not only were the different separations themselves generated using automation, but also the equilibration, rinsing, and preparation of the column for the next set of injections were completely automated. By using this approach, a wide range of mobile phase polarities along with a wide diversity of column stationary phases can be evaluated.

The benefit of this automated process is that numerous combinations of solvent and stationary phase polarities can be evaluated with a minimum amount of operator intervention. The resulting data offer a wide range of possible selectivities from which to choose (10). In fact, the comparison of the chromatographic separation options in each mobile phase with up to six columns may be thought of as a "selectivity survey" from which an appropriate

column and mobile phase combination may be selected. Another benefit of this automated process is that the data necessary to assemble the selectivity survey is conveniently collected overnight, reducing the investigational process to typically two days or less.

In this study, the automatic generation of selectivity surveys is applied to the separation of two mixtures, one containing basic drugs and the other containing a group of herbicides. The development of isocratic separations will be demonstrated without the use of expensive method development software programs by simply using a logical approach. Thus, automatically investigating selectivity variations resulting from changes in both organic solvent type and column type are possible. The process is experiential and allows interaction of the operator at any time, if needed, to override or intercede in the activity.

The objective of our study was to separate four basic drugs in the shortest time possible (under 5 min) with maximum resolution between the critical pair(s). If this goal was not possible, the time could be increased to 10 min, if it was necessary to obtain resolution. In this example, the method was developed at low pH using a phosphate-buffered mobile phase. Operation at low pH suppresses the ionization of the surface silanols, thus, reducing any interactions with the underlying silanols, offering good peak shapes, and robust methods especially, with basic analytes (11). The selectivity surveys (10) that are generated assist the researcher in making the appropriate column/mobile phase choice. Although this automation was used to develop an isocratic separation, it also works equally well for the development of gradient methods.

It is important to emphasize that in this work, certain variables were fixed (pH, buffer type, buffer concentration, and temperature), but the stationary phase and various organic solvent combinations were varied. This choice of conditions may not be appropriate for all applications. For our separation development, however, this was the appropriate path forward because it was based upon our philosophy of achieving a separation with minimum silanol contributions to retention. For another investigator who has a different philosophy, other variables could be examined automatically. For example, if the solvent type and phase were fixed and the column supplier and pH were variables, the system could be programmed to investigate the use of different pH and buffers on columns from different suppliers.

Experimental

Instrumentation and reagents

All experiments were performed on an Agilent 1100 liquid chromatograph (Wilmington, DE) equipped with quaternary pump, autoinjector, heated solvent compartment at room temperature, and diode array detector. The flow rate was set at 2.0 mL/min, and the detector was set to 254 nm. Injections of 1 µL were made for each sample.

The HPLC was equipped with a 6-port SSV (Agilent, part no. G1160A) and connecting tubing (Agilent, part no. G1160-68706) for the low pressure switching. A 12-port high-pressure CSV (Agilent, part no. G1159A) was used to switch between columns using appropriate plumbing (Agilent, part no. G1156-68714). All connections between the columns and CSV were made with 400-× 0.17-mm i.d. (green) tubing. The assembly was facilitated using a column organizer/stand for valves (Agilent, part no. G1383A).

All columns used in these experiments were 4.6 × 75-mm columns packed with 3.5-µm particles for high efficiency. All columns were from Agilent Technologies. The stationary phases were chosen to provide the broadest range of selectivity while maintaining excellent column stability under the low pH conditions used. The following stationary phases were used in this study: Zorbax StableBond-C18 (SB-C18), Zorbax SB-C8, Zorbax SB-Phenyl, Zorbax SB-CN, Zorbax SB-Aq (a proprietary stationary phase used in high aqueous mobile phases), and Zorbax Bonus RP (polar embedded group) (Agilent Technologies).

All organic solvents [methanol (MeOH), acetonitrile (ACN) and tetrahydrofuran (THF)] were HPLC grade (Burdick and Jackson, Phillipsburg, NJ). The aqueous 20mM potassium phosphate buffer of pH 2.0 was prepared in HPLC-grade water. The organic and buffered aqueous mobile phases were chosen so that solubility of both phases was maintained and suitable buffering capacity was available to inhibit the ionization of the silanols on the surface of the silica.

All test samples were prepared by taking 1-mg/mL solutions of each individual component (Sigma-Aldrich, Milwaukee, WI) in the mobile phase buffer and mixing 0.5 mL of each solution make the final test sample. In some cases, the addition of some ACN was required to solubilize each component.

The Version 9.03 Chemstation (Agilent Technologies) was used to control both sets of switching valves. The options available in the software were used to purge the column with the appropriate solvent combinations before each chromatographic run. This operation was to prevent buffer salt from precipitating and to insure that all previous sample and mobile phase components were flushed from the column.

Method development steps

Having fixed certain variables (as mentioned earlier) and deciding that the stationary phase and solvent type were to be varied, the initial task in the development of the separation was to find the appropriate blend of a single mixture of organic–buffer concentration necessary to attain the best resolution of the sample components in the shortest time. Because an aqueous buffer solution mixed with the three popular HPLC organic solvents (MeOH, ACN, or THF) was to be investigated, the initial separation was developed in the weakest organic solvent, MeOH. The most appropriate binary MeOH–buffer mobile phase was determined by "trial-and-error" (12), starting with a 90:10 (v/v, MeOH–buffer) and making the mobile phase sequentially weaker by increasing the buffer content of the mobile phase in discrete increments of 20% until an appropriate separation was attained. If necessary, the final mobile phase was adjusted in 2–5% increments to attain the closest separation to the desired goal.

Once the initial separation was attained in the MeOH-containing mobile phase, it was used on the other columns to determine whether selectivity enhancements could be observed. Following this, the determination of the isoeluotropic mobile phases containing ACN and THF organic solvents were made.

These mobile phases were subsequently evaluated on all of the stationary phases. Isoeluotropic mobile phases have the same solvent strength and can be calculated from values reported in the literature (13–18). The values could also be estimated using a nomograph (18). In this work, the nomograph was used because the isoeluotropic calculation is only an estimation of equal solvent strength, and the nomograph was an easier and more convenient approach.

In each isoeluotropic mobile phase, a neutral compound should have approximately the same retention on each type of phase. Of course, an isoeluotropic mobile phase is determined by an estimate of solvent strength and is not a rigorous value, therefore there may be some variations in retention. The importance of using isoeluotropic mobile phases is that secondary interactions may be introduced that cause selectivity to vary from one mobile phase to another and, hence, possibly improve the separation (16).

The HPLC instrumentation was used to generate the mobile phase compositions. Because it was desirable to keep the ionic strength constant over different solvent ratios, the solvent reservoir bottles contained a premix of buffered water and organic solvent. Thus, the buffer concentration was held constant by using reservoirs that contained premixed solutions (v/v) of 90:10 and 10:90 as the A and B mobile phases, respectively. The steps to attaining the selectivity survey were: Step 1, start with the SB C8 column and 90% MeOH and 10% 20mM potassium phosphate buffer (pH 2.0); Step 2, decrease the amount of MeOH by 20% increments; Step 3, determine an appropriate MeOH concentration for the initial separation; Step 4, run sample on all columns using the chosen MeOH mobile phase; Step 5, estimate the isoeluotropic mobile phase containing ACN; Step 6, run sample on all columns using the isoeluotropic ACN mobile phase; Step 7, estimate the isoeluotropic mobile phase containing THF; Step 8, run sample on all columns using the isoeluotropic THF mobile phase; and Step 9, evaluate the Selectivity Survey for the "best" chromatogram(s).

Note that this approach starts with a C8 column and differs from other method development strategies that use a C18 column. The C8 was used based upon previous experience that the C8 column had similar selectivity to a C18 but showed less retention. This lower retention allowed for shorter run times in the initial steps of this process.

Also, it should be pointed out that Steps 1–3 have often been performed manually. However, Steps 1 and 2 can now be done automatically with the use of the switching valves. Step 3 may require some operator intervention to decide when an appropriate separation is achieved or if more mobile phase combinations require more evaluation. Steps 5 and 7 require calculations or estimations from the nomograph (18), whereupon all remaining steps may be programmed to operate automatically. Often these automatic runs are made overnight augmenting the scientist's effort and supplying a significant amount of selectivity information so that the best column–mobile phase composition can be quickly chosen.

Our experience is that once all steps are completed, the operator can view all chromatograms and from this selectivity survey make the decision as to which mobile phase–column combination is the best for the final method. However, if this decision cannot be easily made based upon the selectivity survey data, the operator has the option to do further fine-tuning of the method development activity using small mobile phase adjustments. It should be pointed out that the general approach is flexible so that, at any time, the operator may intercede and insert intelligent options if inappropriate results are obtained. For example, if no mobile phase tested demonstrated the desired resolution, but one column and solvent combination exhibited good peak shape with partial resolution, the operator could pursue that column with different mobile phase strengths to see whether a final separation of appropriate quality could be attained.

Results and Discussion

Before beginning any method development studies, it is important to verify that the CSV and connecting tubing do not contribute significantly to loss in chromatographic performance of the entire system, including the columns. A simple test was performed by injecting 1 mg/mL of toluene into a 60:40 mixture of methanol–phosphate buffer. The test was performed on the HPLC system without the valve and connecting tubing (column only) and with the valve, tubing, and column in place. A drop in efficiency and increase in peak width would be an indication there was an effect of the added volume on performance; this would negate the desire for highly efficient columns.

The data showed that a 4.6- × 75-mm column in the HPLC system without the CSV and associated tubing had a peak width at half-height of 0.105 min and an efficiency of 13,500 plates at 2.0 mL/min. No change in the measured peak width or efficiency was observed after the valves and associated tubing were inserted into the system. This reflected the fact that the added dead volume of the CSV and connecting tubing did not impact the system performance. Therefore, the assem-

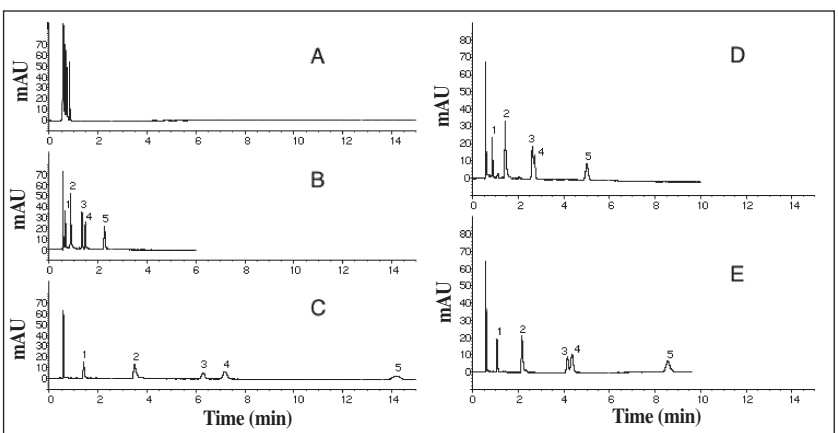

Figure 1. Optimizing the multicomponent mixture using methanol–buffer mobile phase concentration on an SB-C8 column (Steps 1–3). Mobile phase composition of percent methanol: (A) 90%, (B) 70%, (C) 50%, (D) 60%, and (E) 55%. The content of the multicomponent test mixture was propanolol (peak 1), butyl paraben (peak 2), amitriptyline (peak 3), naphthalene (peak 4), and acenaphthene (peak 5).

bled column-switching HPLC system is appropriate for the column dimensions chosen, and the method development activities can be implemented without fear of poor system performance.

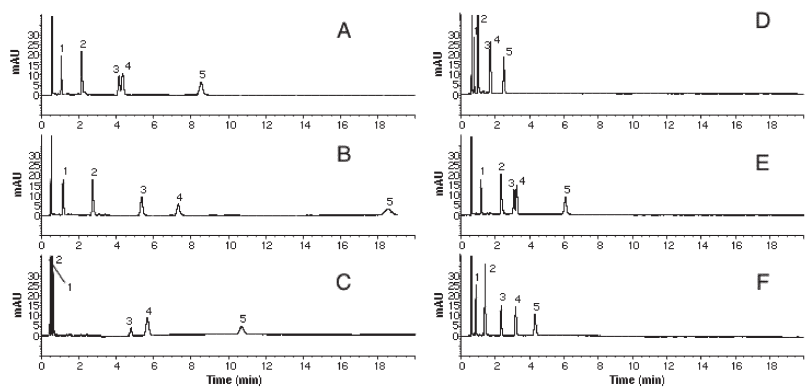

Figure 2. Chromatograms obtained using the optimal 55% MeOH on all columns (Step 4). Columns used: (A) SB-C8, (B) SB-C18, (C) Bonus RP, (D) SB-CN, (E) SB-Phenyl, and (F) SB-Aq. Peak identities are the same as in Figure 1.

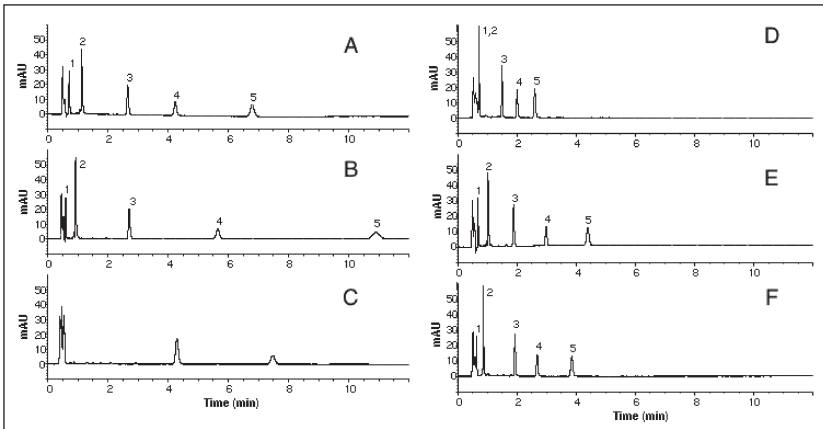

Figure 3. Multicomponent sample run on all columns using isoeluotropic mobile phase of ACN (45%) (Step 5–6). Columns used: (A) SB-C8, (B) SB-C18, (C) Bonus RP, (D) SB-CN, (E) SB-Phenyl, and (F) SB-Aq. Peak identities are the same as in Figure 1.

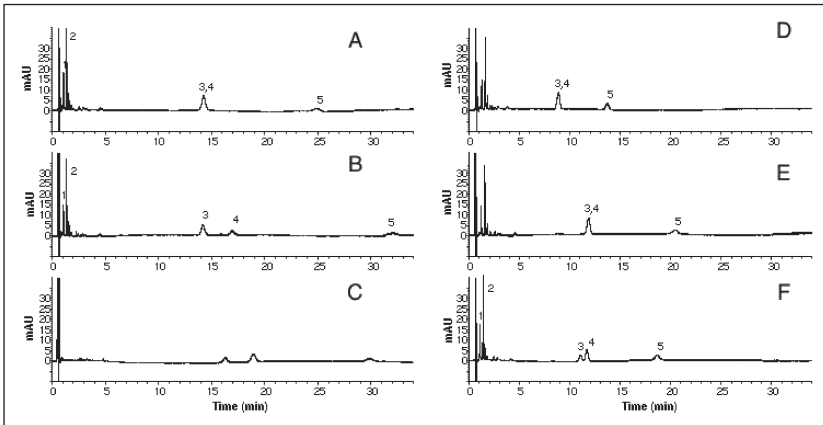

Figure 4. Multicomponent sample run on all columns using isoeluotropic mobile phase of THF (32%) (Step 7–8). Columns used: (A) SB-C8, (B) SB-C18, (C) Bonus RP, (D) SB-CN, (E) SB-Phenyl, and (F) SB-Aq. Peak identities are the same as in Figure 1.

Multicomponent test mixture

To evaluate the feasibility of this approach to the method development activity, a multicomponent sample containing both polar and nonpolar compounds was chosen. The content of the multicomponent test mixture was propanolol, butyl, paraben, amitriptyline, naphthalene, and acenaphthene. By achieving success in developing a separation method for this sample, it would confirm that our strategy could be used for more challenging samples. Our goal for the method was to have baseline resolution of all of the peaks in a minimum amount of time.

The chromatograms resulting from Steps 1–3 are shown in Figure 1. These are used to determine the initial selection of the best methanol–buffer mobile phase on the C8 stationary phase. At 50% methanol, the peaks of the component mixture are separated, but the run times are too long. Increasing the methanol concentration to 55% decreases the resolution and reduces the analysis time while still providing an adequate separation. Further increasing the methanol concentration to 60% shows that the separation begins to degrade as peaks begin to overlap.

Step 4 in the development procedure is to use the 55% methanol mobile phase to determine the type of separation that can be obtained with each of the other stationary phases in the chosen group of column options. This is shown in Figure 2. The SB-CN, SB-Phenyl, and the SB-Aq columns show the potential of a good separation in a minimum amount of time. Further development work is desirable to investigate attaining the best separation.

Step 5 is to determine the isoeluotropic amount of ACN using the nomograph (18) and use this solvent strength as the mobile phase on all of the columns (Step 6). The ACN equivalent to the optimal 55% methanol was determined to be 45%. Analyzing the multicomponent mixture with this mobile phase resulted in the chromatograms shown in Figure 3.

As expected, the retention behavior using the ACN mobile phase was quite similar to those obtained with the methanol-containing mobile phase. The SB-Phenyl and SB-Aq columns provided the best separations, with a slight edge to the SB-Aq column because of the shorter analysis time. The SB-CN also showed suitable separation but required further optimization.

Determining that the isoeluotropic concentration of THF was 32% (Step 7), the sample was run on all six columns (Step 8) using the THF mobile phase. This resulted in the chromatograms shown in Figure 4. Again the SB-CN, SB-Phenyl, and SB-Aq columns provided the best separations in a reasonable amount of time; the SB-Aq column,

perhaps, showing the best separation in this mobile phase.

Except for Steps 1–3 and the calculation of the isoeluotropic concentrations of the various mobile phases, all of the data were gathered overnight. Once all of the data is collected, the analyst may intercede and make decisions for further optimization. From the selectivity survey of the chromatograms obtained with MeOH, ACN, and THF, the best separation can be determined for the requirements of the method (Step 9). Selecting ACN as the organic component was chosen as the best mobile phase. However, slight adjustment of the concentration of the mobile phase was needed with each of the three columns to produce the best resolution of the components, as shown in Figure 5.

From the data shown, the three best separations were on the SB-Phenyl, SB-CN, and SB-Aq columns with the phenyl column having the shortest analysis time. However, if one were interested in the resolution of minor impurity peaks, a different choice could be justified.

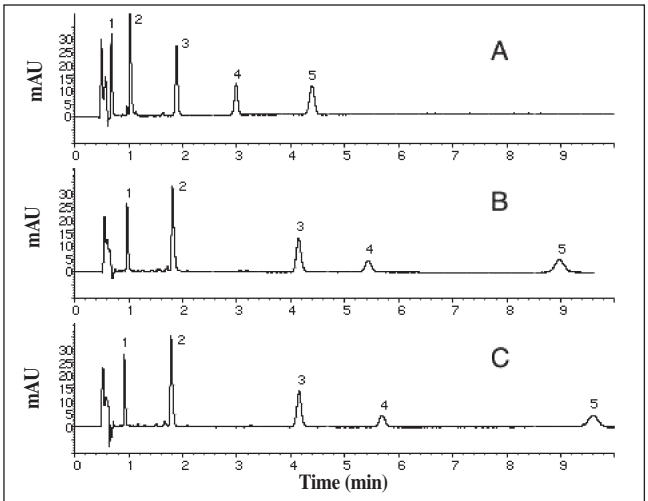

Figure 5. The best solvent–column combinations for separation of the multicomponent sample (Step 9). Column and mobile phase composition: (A) SB-Phenyl using 40% ACN, (B) SB-CN using 35% ACN, and (C) SB-Aq using 30% ACN. Peaks are the same as in Figure 1.

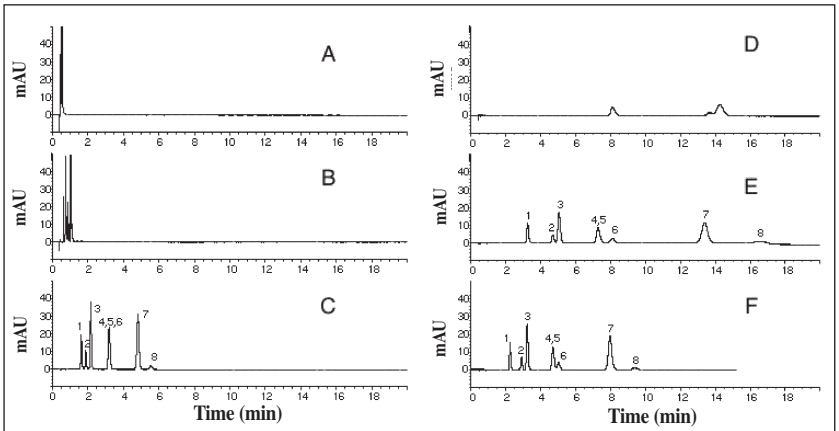

Figure 6. Optimizing the herbicide sample using methanol–buffer mobile phase concentration on an SB-C8 column (Steps 1–3). Methanol composition in percent of total volume: (A) 90%, (B) 70%, (C) 50%, (D) 30%, (E) 40%, and (F) 45%. The order of elution of the herbicides was prometon (peak 1), tebuthiuron (peak 2), prometryne (peak 3), atrazine (peak 4), bentazon (peak 5), propazine (peak 6), propanil (peak 7), and metolachlor (peak 8).

Herbicides

Having shown that the method development steps outlined in the Experimental section work well for developing an isocratic method for a generic test mixture, the methodology was applied to a more difficult sample of eight herbicides. The order of elution was: prometon, tebuthiuron, prometryne, atrazine, bentazon, propazine, propanil, and metolachlor. Again, our goal was to have baseline separation for as many of the components as possible in the minimum of time. The minimum analysis time was to be 10 min or less.

Step 1 in the process was to begin by injecting a sample using a mobile phase mixture of a 90:10 ratio of methanol–phosphate buffer solution on an SB-C8 column. The percentage of methanol was decreased by 20%, and the sample was injected again. This procedure is repeated until the desired separation is observed. The mobile phase concentration may then be further optimized or "fine tuned" by making small incremental changes in the mobile phase composition until the best separation is obtained. Figure 6 shows the chromatograms obtained with the optimum mobile phase defined, which in this case was 45% methanol.

At this point, the separation was not sufficient for the desired objective. However, further work did not improve the separation quality, exhibited by the coelution of two peaks on the SB-C_8 column. Therefore, it was decided to continue with further method development to see if another column using a methanol mobile phase or another isoelutropic mobile phase would result in improved separation.

Step 4 in the process was to use the optimized methanol concentration on all of the different columns. Figure 7 shows the separation on all the columns using the optimum methanol concentration of 45% methanol as determined in Step 3 (Figure 6). Here we found that other columns did indeed resolve an additional peak.

Some of the columns (such as the SB-CN, SB-Phenyl, and SB-Aq) showed promise as potential columns. Some of the peaks were not well resolved using MeOH. However, mobile phase adjustment on these phases might result in a suitable separation. Nevertheless, in keeping with our strategy of creating a selectivity survey before final mobile phase adjustment, we proceeded to evaluate the other common solvents and column combinations.

Steps 5 and 6 in the process were to determine the isoeluotropic amount of acetonitrile and use this as the mobile phase on all columns. In this example, the amount of acetonitrile was determined to be 37%. Analyzing the herbicide sample under these conditions showed the results shown in Figure 8.

Even though, in theory, the isoeluotropic amount of ACN should produce a similar retention as methanol, we see that there can be some interesting differences both in analysis time and selectivity. ACN appeared to have different secondary contributions to solvation of the analyte and stationary phase compared with methanol. The ACN solvent also had the added benefit of lower viscosity that lead to lower system pressure. Here again, the SB-CN, SB-Phenyl, and SB-Aq

appeared to be good columns for this separation in this mobile phase.

Proceeding on and continuing the strategy using THF as the organic component of the mobile phase in Steps 7 and 8, it was determined that the isoeluotropic amount of THF was 26%. Running the herbicide sample under these conditions on all columns produced the results shown in Figure 9.

Here again, it is seen that using the isoeluotropic amount of THF in the mobile phase offered some unique selectivity not seen with the other organic solvents. In this case, the SB-Aq column showed the best separation with a short analysis time.

After examining all of the chromatograms, the best column–mobile phase combinations were selected. Figure 10 shows the best separations from the selectivity surveys of all the conditions tested. The SB-Phenyl and SB-Aq with ACN mobile phase showed good resolution of all components in 6 min or less. The SB-Aq column in THF mobile phase also provided acceptable resolution and analysis time. However, in the THF mobile phase there was a dramatic reversal of peaks 7 and 8, demonstrating the unique role of a mobile phase can play in determining selectivity. In this case, the unique selectivity for propanil and metolachlor that was observed in the THF mobile phase was not seen when using the other organic solvents (Step 9).

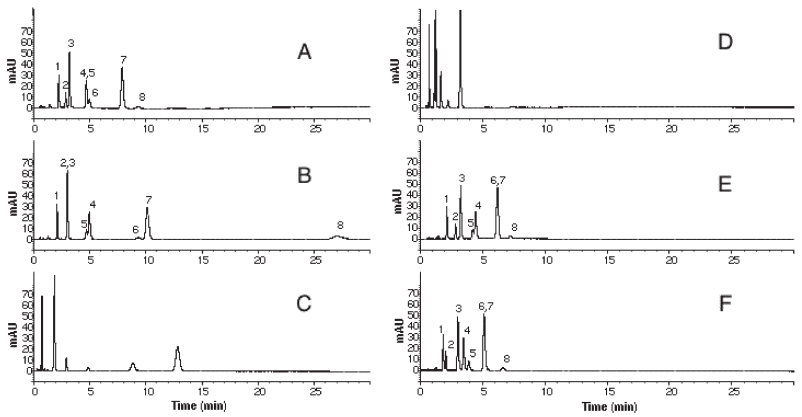

Figure 7. Herbicide sample run on all columns using optimum 45% methanol concentration in mobile phase (Step 4). Columns used: (A) SB-C8, (B) SB-C18, (C) Bonus RP, (D) SB-CN, (E) SB-Phenyl, and (F) SB-Aq. Peak identities are the same as in Figure 6.

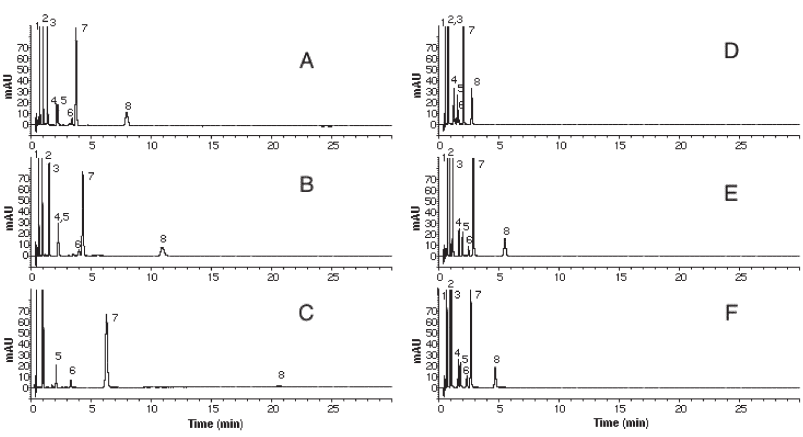

Figure 8. Herbicide sample on all columns using isoeluotropic mobile phase of ACN (36.6%) (Steps 5–6). Columns used: (A) SB-C8, (B) SB-C18, (C) Bonus RP, (D) SB-CN, (E) SB-Phenyl, and (F) SB-Aq. Peak identities are the same as in Figure 6.

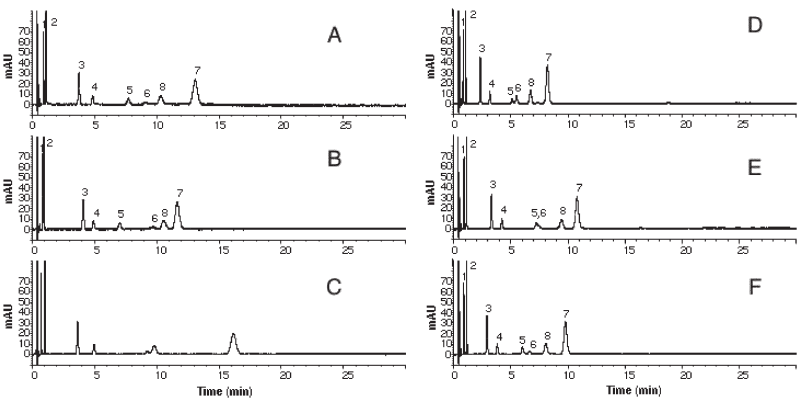

Figure 9. Herbicide sample on all columns using isoeluotropic mobile phase of THF (26%) (Steps 7–8). Columns used: (A) SB-C8, (B) SB-C18, (C) Bonus RP, (D) SB-CN, (E) SB-Phenyl, and (F) SB-Aq. Peak identities are the same as in Figure 6.

Conclusion

The outlined liquid chromatographic method development strategy is an effective way of developing isocratic methods in a short time. Changes can easily be made with solvents in combination with column switching valves controlled by the software. Furthermore, software control allows unattended operation with column and solvent switching, although some operator intervention is needed when choosing mobile phase concentrations.

Changing the mobile phase components and column selectivity can be a powerful way of optimizing a separation. Methanol with a 20mM phosphate buffer is a good first choice for the mobile phase. Switching to other organic solvents, such as ACN, can reduce viscosity to provide a unique selectivity for some compounds. The C8 column is a good stationary phase for initial scouting as its selectivity is similar to a C18 stationary phase. However, the C8 provides shorter retention and, thus, faster scouting for the initial separation. By determining the selectivity survey, very wide ranges of selectivity options are available for the final method. This work also demonstrates that if only a C18 column was used, the "best" final method would be artificially constrained and may not reflect the truly "best" separation.

In fact, what was somewhat surprising was the fact that the best separation did not use a C18

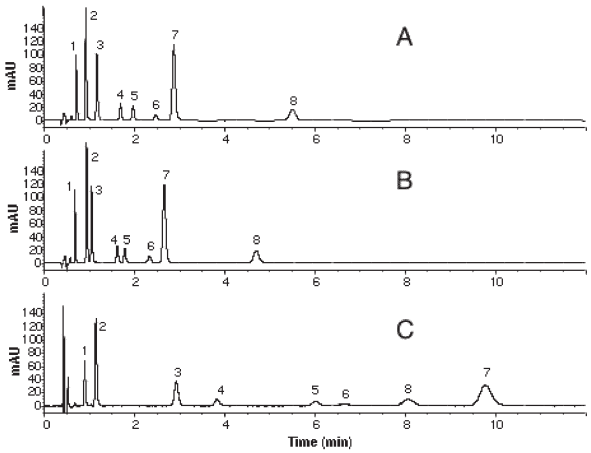

Figure 10. The best solvent–column combinations for separation of the herbicide sample (Step 9). Column and mobile phase composition: (A) SB-Phenyl using 36.6% ACN, (B) SB-Aq using 36.6% ACN, and (C) SB-Aq in 26% THF. Peaks are the same as in Figure 6.

phase. Yet, the C18 is the most popular stationary phase used. In this work the C18 column always had longer retention times. Interestingly, the SB-Aq phase often had a similar selectivity to the C18 phase with appropriate resolution but with a much shorter analysis time. The SB-C18 column had resolution but always with longer analysis time, which eliminated it from the "best" method category. In addition to the SB-Aq, the other phases such as SB-CN and, SB-Phenyl provided unique selectivity for developing these separations that were also better than those on the C18 column.

References

1. J.L. Glajch and L.R. Snyder, Eds. *Computer-Assisted Development for HPLC.* Special issue of *J. Chromatogr.* **485:** 1–675 (1989).
2. L.R. Snyder. Classification of the solvent properties of common liquids. *J. Chromatogr. Sci.* **16:** 223–34 (1978).
3. L.C. Sander and S.A. Wise. Evaluation of shape selectivity in liquid chromatography. *LC-GC* **8(5):** 378–88 (1990).
4. J.J. DeStefano, J.A. Lewis, and L.R. Snyder. Reversed-phase high performance liquid chromatography method development based on column selectivity. *LC-GC* **10(2):** 130–39 (1992).
5. P.E. Antle, A.P. Goldberg, and L.R. Snyder. Characterization of silica-based reversed-phase columns with respect to retention selectivity. solvophobic effects. *J. Chromatogr.* **321:** 1–32 (1985).
6. J.L. Glajch, J.C. Gluckman, J.G. Charikovfsky, J.M. Minor, and J.J. Kirkland. Simultaneous selectivity optimization of mobile and stationary phases in reversed-phase liquid chromatography for isocratic separations of phenylthiohydantoin amino-acid derivatives. *J. Chromatogr.* **318:** 23–39 (1985).
7. R.D. Ricker, B.A. Bidlingmeyer, and J.J. Kirkland. *Method Development Flow Chart.* Publication no. 5968-8393EUS, Agilent Technologies, Wilmington, DE, 2000.
8. J.J. Kirkland and J.W. Henderson. Reversed-phase HPLC selectivity and retention characteristics of conformationally different bonded alkyl stationary phases. *J. Chromatogr. Sci.* **32:** 473–80 (1994).
9. U. Huber. "Method Development-Solvent and Column Selection with the Agilent 1100 Series Valve Solutions". Publication no. 5988-8347EN, Agilent Technologies Application Note, Wilmington, DE, 2002.
10. B.A. Bidlingmeyer, A.D. Broske, and W.D. Snyder. "Valve switching used for rapid LC method development." Paper number 1380-2, Presented at the Pittsburgh Conference, Orlando, FL, 2003.
11. L.R. Snyder, J.J. Kirkland, and J.L. Glajch. *Practical HPLC Method Development.* John Wiley & Sons, New York, NY, 1997.
12. B.A. Bidlingmeyer. *Practical HPLC Methodology and Applications.* John Wiley & Sons, New York, NY, 1993.
13. L.R. Snyder, J.W. Dolan, and J.R. Gant. Gradient elution in high-performance liquid chromatography I. Theoretical basis for reversed-phase systems. *J. Chromatogr.* **165:** 3–30 (1979).
14. P.J. Schoenmakers, H.A.H. Billiet, and L. deGalan. Influence of organic modifiers on the retention behavior in reversed-phase liquid chromatography and its consequences for gradient elution. *J. Chromatogr.* **185:** 179–95 (1979).
15. P.J. Schoenmakers, H.A.H. Billiet, and L. deGalan. Systemic study of ternary solvent behavior in reversed-phase liquid chromatography. *J. Chromatogr.* **218:** 261–84 (1981).
16. L.R. Snyder, J.L. Glajch, and J.J. Kirkland. Theoretical basis for systematic optimization of mobile phase selectivity in liquid-solid chromatography: solvent-solute localization effects. *J. Chromatogr.* **218:** 299–326 (1981).
17. J.W. Dolan. Shortcuts for LC measurements. *LC-GC* **5(12):** 1030–32 (1987).
18. L.R. Snyder, J.J. Kirkland, and J.L. Glajch. *Practical HPLC Method Development.* John Wiley & Sons, New York, NY, 1997, p. 239.

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