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A virtual-modeling and multivariate-optimization examination of HPLC parameter interactions and opportunities for saving analysis time

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Abstract

The interrelations of parameters in HPLC are very complicated, even in a simple problem. Optimization requires considering all the adjustable parameters in concert, but the amount of work required to do this experimentally is prohibitive. However, if we first choose the selectivity parameters, we can then successfully and rapidly perform a multivariate optimization of the efficiency parameters within a numerical model. By examining this process with a level of detail not normally necessary in routine work, we reveal the complexity of parameter interactions in a simple separation, and the potential for large savings of analysis time by properly balancing parameter values. We show how to reduce a 13 min experimental separation to less than 2 min without utilizing ultra-small particles or pressure beyond the capabilities of an ordinary HPLC instrument. Ultra-small particles will often improve analysis times when the separation is plate-number-limited, but if the particles are smaller than optimal for the required separation, then larger particles will require less analysis time.

Keywords: Optimization; Multivariate optimisation; Modeling; Particle diameter; Pressure; Butylparaben; Propranolol; Naphthalene; Acenapthene; Amitriptyline

1. Introduction

High performance liquid chromatography (HPLC) is often regarded as a mature technique, one with thousands of practitioners successfully solving problems in a large variety of applications. However, because of the difficulty and complexity of experimental optimization, few workers in practice are able to approach the best-possible performance of a separation. The usual guidance available concerning the overall quality of a separation is the expectation based on past performance in the same workgroup rather than any real (or virtual) knowledge of what is actually possible. If there is a business expectation to find reasonable separation conditions within a couple of days, then there are only a dozen or so experiments possible before time runs out.

Nearly every HPLC practitioner is familiar with the relations between flow rate, column dimensions, particle diameter, and plate number, and the relations between particle diameter, flow rate, column dimensions, and pressure. These are easy to accurately model. Practitioners often assume that the logarithm of the retention factor ($\log k$) decreases linearly with increasing modifier concentration in the mobile phase [1]. If all the solutes change their $\log k$ values at approximately the same rate with respect to the modifier concentration, then the selectivity (that is, the values of the separation factors, α, for the peaks compared pair wise, where $\alpha = k_2/k_1$ for any given pair being compared) is also nearly constant with respect to the modifier concentration. While these assumptions are approximately correct, it is the deviations from these expectations and the complicated interrelationships of the effects of these parameters, combined with particle diameter, flow rate, and column dimension effects on the separation performance, that provide us with unexpected opportunities to make performance improvements.

1.1. Optimization

Improving HPLC separations by experimental one-at-a-time tweaking of one or two parameters, without regard to

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parameter interactions or to the influences of other easily adjusted parameters, is commonly practiced. Such efforts performed serially on several parameters may provide improvement in some respect, but additional refinement of the same parameters will often continue leading to even better performance.

Optimization, by contrast, finds the unique combination of values of the adjustable parameters corresponding to the best performance possible for a particular set of requirements. By definition, there is no means to further improve an optimized separation unless, as we will see, the requirements or limits are changed or another parameter is declared adjustable and added to the problem. Thus, the result of an optimization is totally dependent on the goals of the separation, the parameters which are considered adjustable, and the limits or constraints placed on the parameter values.

A robust optimum is within a range of values in the parameter space over which similar results can be obtained. Thus, a robust optimum allows a degree of flexibility and convenience in selecting the values of continuously variable parameters (like flow rate and modifier concentration), but we will see that performance can be compromised by the limited number of choices among discrete parameters like column length and particle size, and that the robustness of an optimum may vary with respect to the individual parameters.

An experimental optimization procedure would take hundreds of experiments to execute, perhaps including experiments requiring very long analysis times or inconvenient parameter values to gain the required knowledge. Numerical modeling is a very efficient alternative to experiment when the model provides the required level of accuracy.

1.2. Summary of our modeling and optimization approach

Our model and methods were disclosed earlier [2,3] and have not been changed. Efficiency parameters, like dimensions and flow rate, are mostly independent of selectivity parameters, such as stationary phase, modifier choice, pH, temperature, etc. At present, we do not include these selectivity parameters in our model. Instead, we screen columns, mobile phases, pH, and temperature experimentally to find a promising combination to take forward into modeling and efficiency optimization. Of course, our ultimate outcome depends strongly on the goodness of our effort in setting the selectivity parameters. This remains a key part of HPLC method development, and expertise in making these choices is highly valued.

We have found that the effects of particle diameter, column dimensions, flow rate, and extra-column effects can be accurately predicted from theory alone. We prefer to use a quadratic function to relate $\log k$ for each solute to modifier concentration [4], and then evaluate the coefficients by a least-squares fit of data from at least four experimental trials spanning the modifier concentration range of interest.

We use a constraint-based multivariate optimization strategy in which the required outcome is expressed among other constraints in the optimization problem [2,3,5–7]. For example, for an assay of components in a product, we can objectively state the resolution required around every peak in the chromatogram. Peaks of no interest can have their required resolution defined as zero. In a limit test we might also state a minimum-required peak height or area and include sample size and concentration among the variables investigated. In a preparative separation we can state goals such as the purity of the target material, a maximum cost, or a minimum production rate. Once the objective business outcome is defined, our next step is to perform a multivariate optimization whose purpose is to minimize or maximize one other secondary but important performance parameter. For example, we can minimize the analysis time while ensuring that the required resolution is achieved. Another possibility is to minimize the analysis cost. The lowest-cost conditions may require a long analysis time in some instances, so a practical analysis-time limit can be specified, if necessary. Other constraints, for example, reasonable limits on pressure, flow rate, mobile-phase composition, particle diameter, column dimensions, etc., can be added to make sure that the solution is practical.

This approach is a significant departure from defining a single objective quality function for the separation [8–16]. Quality functions may sacrifice the resolution around one or more important peaks if the time savings is large, and thereby fail to meet the goals of the separation. The constraint-based approach requires that all the business needs are met, and that anything less is unsuitable.

The purposes of the present work are to examine a relatively simple problem and to more fully reveal the interrelations of the HPLC parameters in this problem. We also will demonstrate the utility of numerical modeling combined with constraint-based multivariate optimization for improving HPLC results with little experimental effort.

2. Experimental

Work was performed using a model 2695 Separations Module (Water Corporation, Milford, MA, USA) equipped with vacuum degassing, a column heater/cooler module, a six-column selection valve, and a model 2996 photodiode array detector. We have not modified this instrument since receiving it from the manufacturer. The additional tubing associated with the column selection valve added extra-column volume to the system not normally present in other model 2695-based systems. The extra-column dimensions are summarized in Table 1.

The column was a Symmetry C18 with 3.5 μm particles, 4.6 mm × 10 cm (Waters Corporation). It had previously been used and may not be representative of new columns. The mobile phase was dynamically mixed on-line from buffer (20 mM disodiumhydrogen phosphate adjusted to

Table 1
Extra-column dimensions for valve position 1

Injector	
Loop diameter (in.)	0.020[a]
Sample volume (μL)	10
Inlet tube	
Diameter (in.)	0.009[b]
Length (cm)	136[b]
Outlet tube	
Diameter (in.)	0.01
Length (cm)	158[c]
Detector	
Path length (mm)	10
Volume (μL)	10

[a] The length of the sample loop tube is of no concern because the sample is removed from the tube by backflushing and is exposed only to the length of tube necessary to contain the sample.

[b] A 115 cm length of 0.009 in. i.d. tubing connects the injector to the column selection valve, and a 21 cm length of 0.01 in. PEEK tubing connects the valve to the column inlet. We chose to use the narrower diameter in the calculations and to ignore the broadening in the valve compared to that in the tubing.

[c] This is the total length of two pieces of PEEK tubing connecting the column outlet to the column selection valve and the valve to the detector inlet. We ignored the broadening in the valve compared to that in the tubing.

pH 7.00 with phosphoric acid, bottle A) and methanol (bottle B). All separations were performed with the column oven temperature set to 23 °C. A test solution was made containing the solutes uracil, propranolol, butylparaben, naphathalene, acenaphthene, and amitriptyline, which were typical laboratory-grade materials obtained from a variety of common sources. They were dissolved in mobile phase (approximately 50% methanol) with each solute within the range of 30–70 μg/mL. Experimental separations were conducted at 1 mL/min with mobile phases of 60, 65, 70, and 75% methanol using 10 μL injections. Spectra were recorded from 200 to 300 nm to allow verification of the peak identities.

Modeling [2,3] was accomplished within an Excel 2002 workbook (Microsoft Corporation, Redmond, WA, USA). HPLC performance is highly dependent on the extra-column volume and dimensions, so delays and broadening caused by extra-column effects are included in the model. The approach allows us to specify the resolution required around each peak irrespective of changes in relative peak spacing or elution order. We usually specify constraints on pressure, flow rate, and column dimensions, but additional constraints can easily be added if required by the situation. Optimization is performed using the Excel's Solver add-in. In the present work, we specified the required resolution and then minimized the necessary analysis time, where analysis time was arbitrarily defined as the retention time of the last peak. (We could have just as arbitrarily defined this as the retention time of the last peak plus half its width, or retention time of the last peak plus one minute or some other time increment; these differences would not have generated significant changes in the outcomes of our calculations.) Except where noted, virtual injection volumes were 10 μL.

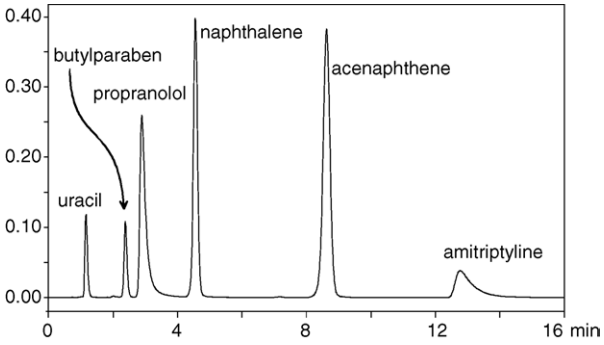

Fig. 1. Actual chromatogram, 75% methanol, 1 mL/min, 225 nm.

3. Results and discussion

Fig. 1 is one actual chromatogram from among the data collected for the modeling. This one was acquired with 75% methanol and was the shortest in duration. The corresponding experimentally determined retention factors used to define the model are summarized in Fig. 2a. The retention of uracil was not constant but ranged from 1.16 min (with 75% methanol) to 1.37 min (with 60% methanol). The first

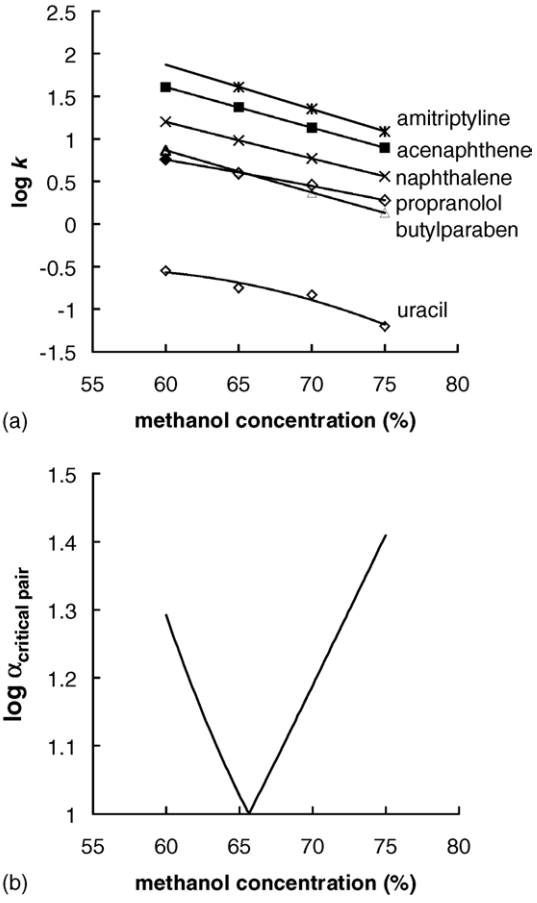

Fig. 2. Retention data used to make the model. (a) log *k* values vs. methanol concentration in the mobile phase. (b) log of the critical-pair separation factor vs. methanol concentration in the mobile phase. The critical pair is butylparaben and propranolol over the range of this figure.

consistent disturbance in the chromatograms occurred at 1.10 min, and we used this value for calculating retention factors. Because uracil was eluted so near the void volume, there is considerable relative error in the calculated uracil retention factors, but the errors are very small in calculating uracil retention times. For the remaining solutes, the plots of log k versus methanol concentration are sufficiently linear in this work that a linear retention model would have been adequate. The largest difference between predicted retention times comparing linear with quadratic fits was 0.05 min for propranolol, the most curved of the well-retained solutes. We projected from the three highest methanol concentrations (which were acquired first) that amitriptyline would be retained over 70 min with a k value around 75 when using 60% methanol. This datum would add little value to the model, so we did not obtain this experimental point for amitriptyline, and modeled this solute linearly with the remaining three points.

Butylparaben and propranolol switched elution order within the data range, with co-elution appearing to occur at 66% methanol. This elution order switch leads to two regions of methanol concentration, one below and the other above 66%, where the selectivity may be sufficient to achieve reasonable separations of all the solutes. This is shown objectively by the selectivity window diagram in Fig. 2b. We cannot tell at this point which of these two regions will give the better solution to our problem. It is often not possible for a generalized reduced gradient optimization tool, like Solver, or other optimization approaches based on projecting from previous results, like Simplex methods [17], to recognize local optima and, in this case, the existence of an alternate and perhaps superior solution located in a direction of the parameter space where performance is decreasing. Therefore, when multiple regions that may contain local optima appear to exist within the parameter space of the model, it is best to virtually explore each region separately. Each takes only a few seconds of calculations; however, for illustrating the behavior of the model and the interdependencies of the parameters, we will look at the results much more thoroughly than would be required during a practical method development and optimization effort.

3.1. Behavior of the model, and optimization issues

We defined the resolution goals of the separation to provide a minimum resolution of 2.0 around every peak except uracil, which was included only as a void volume marker. Therefore, the required resolution for uracil was set at zero. We then focused our efforts on minimizing the analysis time while achieving these stated resolution goals. We also set constraints, when necessary, for maximum pressure, maximum flow rate, column dimensions, particle diameter, and in some cases maximum methanol concentration in the mobile phase. The experimental pressure averaged 17.9 MPa (2600 psi) at 1 mL/min during data collection, and that was used to calculate the pressure under other conditions encountered virtually during the modeling and optimization calculations.

It is highly instructive for understanding parameter interrelations to perform a series of optimizations while varying an important constraint. The results are then most easily summarized by plotting the locus of a performance factor of the individual optimized solutions, and some of the associated operational and other performance parameter values for each optimum, as a function of the constraint being varied. Fig. 3 shows calculation results meeting our

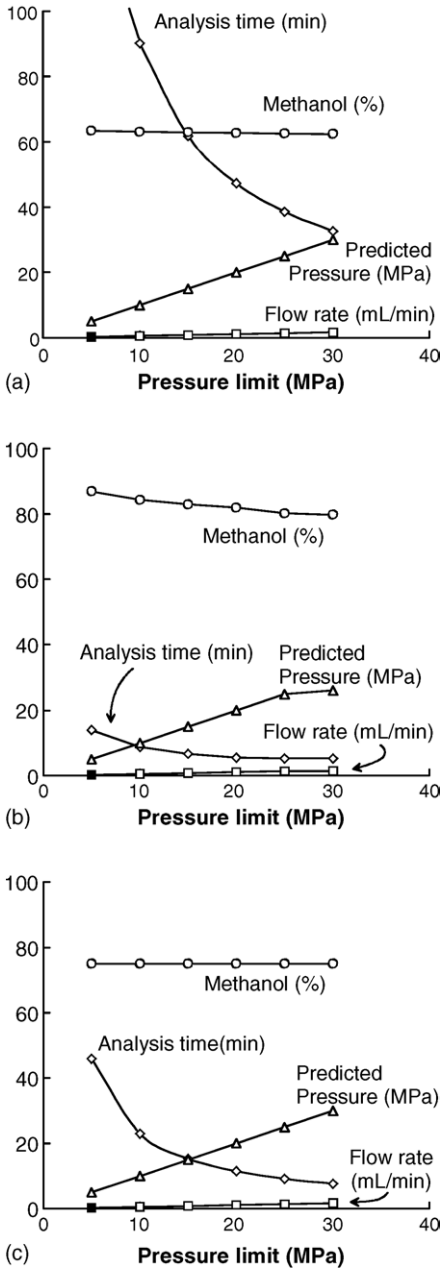

Fig. 3. Loci of optimal analysis times as a function of the pressure limit, shown with the corresponding values of flow rate, pressure, and methanol concentration. The particle diameter and column dimensions are fixed at 3.5 μm and at 4.6 mm × 10 cm, respectively, and 2.0 is the minimum allowed resolution among peaks of interest. Optima found (a) below 66% methanol, (b) above 66% methanol, and (c) between 66 and 75% methanol. Note that here all the optima require 75% methanol.

resolution requirements for the 10 cm long column, packed with 3.5 μm particles, as a function of the pressure limit.

Fig. 3a shows a locus of local optima of analysis times and the associated methanol concentrations, flow rates and predicted pressures for these optima. In this figure the methanol concentration was allowed to vary below 66%, and the flow rate was allowed to vary up to 5 mL/min as long as the pressure stayed below the pressure limit. The predicted pressure of each optima matches the pressure limit, so pressure is always a limiting constraint in this case. Therefore, it is not surprising that the analysis times decrease as more pressure is allowed: the best possible analysis time is 175 min when the pressure limit is 5 MPa, but continuously improves to 32.6 min as we raise the pressure limit up to 30 MPa. The flow rate and pressure are in constant ratio as we would expect since the column dimensions are fixed in this example. The methanol concentration decreases from 63.3% at 5 MPa to 62.4% at 30 MPa. This is a small but, importantly, a counterintuitive change (weakening the mobile phase to produce a faster analysis as the analysis time decreases).

Fig. 3b shows the locus of optima above 66% methanol. These analysis times are much faster than in Fig. 3a, and confirm that the optima below 66% methanol are local optima (at least some of the time). Above 66% methanol, only 14 min are required for the separation with a 5 MPa pressure limit, and this decreases to 5.29 min as the pressure limit is raised to 30 MPa. Note that only 26 MPa pressure was required when the pressure limit was set to 30 MPa. The methanol concentration again counterintuitively weakens as the analysis time is shortened.

We have two potential problems with the virtual results in Fig. 3b: first, the percent methanol in these solutions is well outside the range of our initial data, and there is no certainty that we can extrapolate our model into this methanol concentration range with sufficient accuracy; and second, operating the mobile phase with such high methanol concentration may precipitate buffer and cause problems with mobile phase delivery. Therefore, it may be prudent to further constrain the methanol concentration to no more than 75% since this value is both within our actual data range, and it caused no precipitation problems. A plot of optima using this methanol concentration limit is shown in Fig. 3c. The predicted analysis time is 46 min for a 5 MPa pressure limit. This improves smoothly to 7.7 min as the pressure limit is increased to 30 MPa. Every solution requires the maximum allowed methanol concentration, 75%.

The behavior in Fig. 3 may seem predictable in hindsight since, in all the calculated optima except the 30 MPa result in Fig. 3b, the flow rate was as high as the pressure would allow, and the methanol concentration was at the highest value that could resolve the peaks at their specified resolution values at that flow rate. However, in Fig. 3b, the predicted pressure is only 26 MPa when the pressure limit is set to 30 MPa; thus, the pressure is no longer a limiting constraint, and allowing even higher pressure provides no further improvements (because the additional allowed pressure is not utilized). In this case, a faster analysis requires spending the additional pressure not only on flow rate but on lengthening the column because the plate number has become limiting.

3.2. Adding column length to the optimization

Each time we add another variable to the optimization, the complexity of the problem greatly increases. Yet, when additional parameters are included in the exercise, the outcome can be improved further. The correct column length for a particular separation, like any other parameter value, depends on all the requirements, which parameters are allowed to change, and constraints. Fig. 4a shows the results for 3.5 μm particles when the locus of analysis time optima is plotted as a function of column length for methanol concentrations above 66% and with a pressure limit of 30 MPa. Instead of calculating optima only at the commercially available column lengths, we included results at numerous intermediate lengths in order to clearly see the interactions among the parameters. Keep in mind that such plots represent loci of optimal solutions depending on the column length, the independent variable in this representation. The best possible column length is 11.1 cm for a 30 MPa pressure limit. The corresponding values of the other parameters are 1.51 mL/min and 81.9% methanol, and the analysis takes 4.55 min. Deviations in either direction from this column length require longer analysis times. The best commercially available column length to use appears to be 10 cm with the considerations made so far, and this column length gives the 5.29 min, 26 MPa solution discussed earlier (Fig. 3b).

Fig. 4b is similar except that the methanol concentration was constrained not to exceed 75%. With this constraint the best column length is 9.8 cm, the corresponding analysis time is 7.32 min, the flow rate is 1.71 mL/min, and the pressure is at the 30 MPa limit. A 10 cm commercial column is again the best choice, but gives slightly different values of the other parameters, and increases the analysis time slightly to 7.65 min from the optimum in Fig. 4a.

Fig. 4c shows loci of optima for methanol concentrations below 66% as a function of column length. A 3 cm long column will provide a 5.8 min analysis using 57.5% methanol but requires a 5 mL/min flow rate and over 26 MPa of pressure. This is feasible, but the conditions would be very unusual. We will continue examining the possibilities above 66% methanol.

In ordinary practice, we would usually treat the column length as a continuous variable and simply include it in the optimization calculations; thus, our process returns the best combination of parameter values that minimizes the analysis time while meeting all requirements and constraints for the problem at hand. After thus determining the best-possible column length, we would next optimize for the specific commercially available column lengths on either side of the optimal length, and then choose which to use. Note that as we vary the column length, the other parameters allowed to vary

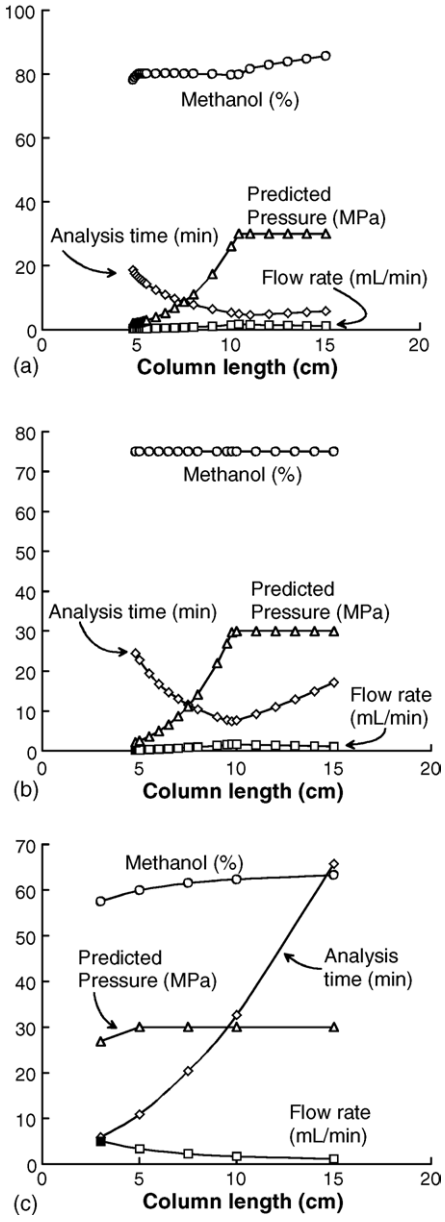

Fig. 4. Loci of optimal analysis times as a function of column length, shown with the corresponding values of flow rate, pressure, and methanol concentration. The particle diameter is fixed at 3.5 μm, and 2.0 is the minimum allowed resolution among peaks of interest. (a) Optima for methanol concentrations above 66% and with a 30 MPa pressure limit. Note that the pressure is not limiting for column lengths below 11.1 cm. (b) Optima for methanol between 66 and 75%. Note that here all the optima require 75% methanol. (c) Optima for methanol concentrations below 66%.

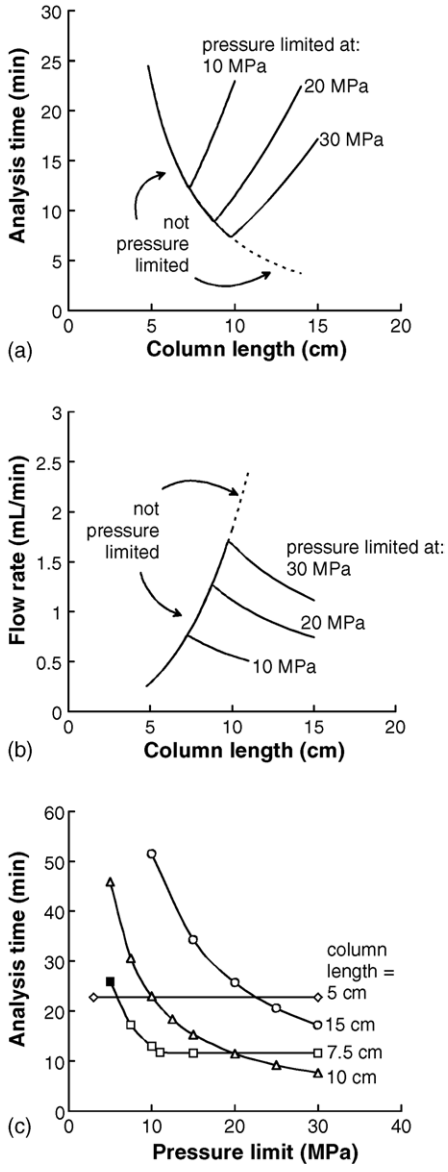

Fig. 5. More details of column length effects for optima calculated in the methanol concentration range between 66 and 75% with a fixed 3.5 μm particle diameter and 2.0 minimum resolution. These results are calculated for an instrument with 0.146 mL of extra-column volume. (a) Loci of optimal analysis times as a function of column length. Note the family of V-shaped loci that are pressure-limit dependent. The left side of each V is common and is not pressure-limited, and the right side is pressure-limited at the values shown. (b) Loci of the flow rates corresponding to the analysis times above. (c) Loci of optimal analysis times as a function of the pressure limit for four different fixed column lengths.

will usually change their values unless they are at a constraint limit.

Fig. 5a further illustrates the relation between analysis time, column length and pressure limit for the 3.5 μm particles for methanol concentrations limited between 66 and 75%. The relationship between analysis time and column length without consideration of a pressure limit is given by the curve segment on the left side of the figure. The various pressure-limited cases intersect this curve producing a V-shaped locus of optima for each pressure limit: At 10 MPa the best column length (corresponding to the bottom of the V) is 7.25 cm, and this would provide a 12.3 min analysis. At 20 MPa the best column length is 8.75 cm giving an 8.5 min analysis, and at 30 MPa the best column length is 9.8 cm and would require 7.3 min. The corresponding flow rates are shown in Fig. 5b. It is clear from these figures that achiev-

ing optimal performance requires careful balancing of the adjustable parameters.

Each pressure limit produces a corresponding minimum analysis time for a column of some specific length as revealed by these figures. When the column length is shorter than the optimal value, the separation is usually plate-number-limited (among possibly other limits) and the flow rate must not exceed the value giving the required number of plates to solve the problem. Improving the analysis time in this case requires lengthening the column if the particle diameter is held constant. When the column length is longer than the optimal value and the modifier is constrained not to go higher, the separation is pressure limited and the flow rate is not allowed to exceed the value giving the maximum pressure. There are more plates than necessary, and excess resolution is produced. Improving the analysis time requires shortening the column if the particle diameter is held constant.

An important practical decision involves selecting a particular commercially available column length (assuming the particle diameter is the same among the columns being considered). Fig. 5a reveals that, because of the relatively narrow V-shaped curves, the optimal analysis times are not very robust with respect to column length. Selecting from among the available column lengths is more easily done with using Fig. 5c, which is another representation of the same calculations except that the pressure limit is changed continuously as the independent variable, and analysis time optima are shown for the available column lengths. There are no feasible solutions meeting the resolution requirements using the 3 cm column (not shown) with methanol concentrations between 66 and 75%. Below about 6 MPa, the 5 cm column will produce the fastest analysis, about 23 min. This requires only 2.55 MPa, so the solution is never pressure-limited in the range of the figure. To go faster we must allow more pressure and lengthen the column. With the pressure limit anywhere between 6 and 20 MPa, the 7.5 cm column is fastest. The locus of optima with this column is pressure-limited below 11 MPa. When higher pressure is allowed, the 7.5 cm column remains plate-number limited and continues to operate at 11 MPa despite the higher pressure limit. To go any faster again requires going to the next-longer column (10 cm) and allowing more than 20 MPa of pressure. This column is always pressure-constrained in the range of the figure, and can produce a 7.6 min analysis at 30 MPa and 1.7 mL/min. The 15 cm column is never the best choice in the pressure range shown here, and would require a pressure limit above 73 MPa (>10,000 psi) before it could improve upon the analysis time of the 10 cm column.

3.3. Extra-column volume effects

The use of ever-diminishing particle diameters in HPLC has greatly increased the relative contribution of extra-column volume to solute peak widths, especially for early peaks in isocratic separations. It is common in the workplace to see newer columns with smaller dimensions experimentally tested or even routinely used on older HPLC instruments with little regard for how much column performance is wasted by extra-column broadening. Specifications for new HPLC instruments have also improved but have not always kept up with the requirements of the columns routinely used today.

The instrument used for this work contains, by our estimate, 0.146 mL of extra-column volume in the sample path. Ideally, the column should be responsible for 100% of the peak broadening experienced, and the rest of the system should contribute none. However, for a peak eluted isocratically with $k = 2$, the pre-column volume in this instrument contributes approximately 25% of the peak variance (that is, the square of the peak standard deviation, a measure of peak width), and the post-column volume contributes over 40%. These figures correspond to the column dimensions and flow rate used to collect the model data. Using higher retention factors reduces the relative contribution of the extra-column broadening, and when $k = 10$ the outlet tube contributes less than 10% in this instrument.

Virtually changing the column inlet and outlet tubes to 40 cm lengths of 0.005 in. i.d. tubing reduces the total extra-column volume to 0.020 mL and reduces the contributions of the inlet and outlet tubes to less than 2% of the peak variance when $k = 2$. Since our critical peak pair is butylparaben and propranolol, and since these elute early in the chromatogram and are greatly affected by extra-column volume effects, removing extra-column volume greatly changes the calculated performance and optima. To adapt our model data, acquired on our actual instrument, to match the dimensions of our virtual instrument with reduced extra-column volume, we subtracted 0.126 min (that is, the time saved by the extra-column volume reduction at 1 mL/min) from every experimental retention time in the model. This correction is necessary in order for the two models to agree in their retention factor predictions.

Fig. 6a shows the locus of optimal analysis times versus the column length for several different pressure limits after virtually reducing the extra-column volumes. Note the large improvement in analysis time compared to Fig. 5. Fig. 6b shows the flow rates corresponding to the conditions in Fig. 6a. With the original, unmodified instrument, the best predicted analysis time was 7.6 min with a 30 MPa pressure limit. But now, if keeping the pressure low is an important concern, it is possible to achieve a 5.2 min analysis with only 4 MPa using a 3 cm column. This saves over 30% of the analysis time and 86% of the pressure compared to the best of the earlier results, and saves about 60% of the analysis time required in the fastest of the experimental chromatograms (Fig. 1). If speed is more important than pressure, Fig. 6c shows that a 5 cm long column could produce an even faster analysis if the pressure limit is above 11 MPa: with a 20 MPa limit the analysis can be done in less than 3 min, and with a 30 MPa pressure limit the time required is less than 2 min. Columns longer than 5 cm are always pressure-

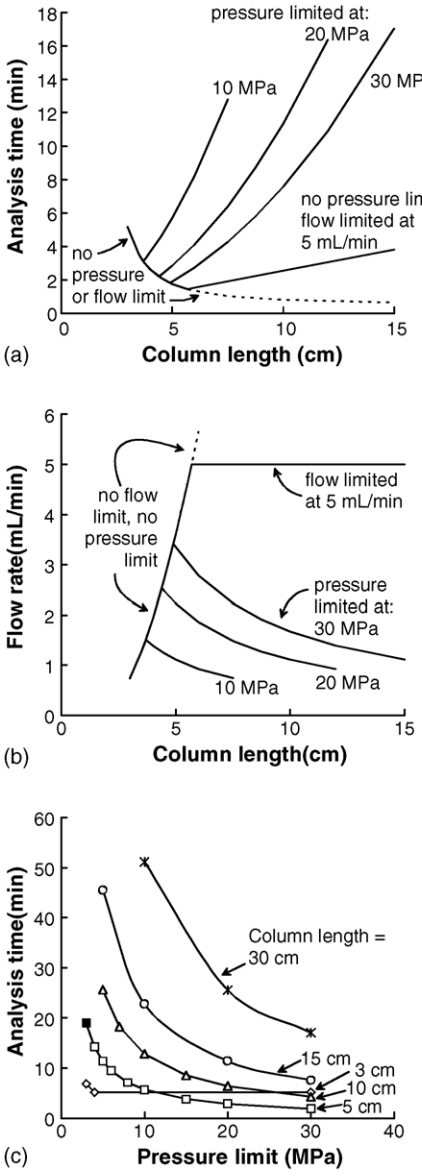

Fig. 6. These are the same kinds of plots as in Fig. 5 except that the extra-column volume was virtually lowered from 0.146 to 0.020 mL and the calculations were repeated. The particle diameter is still fixed at 3.5 μm and 2.0 is the minimum resolution allowed. (a) Loci of optimal analysis times as a function of column length. Note the improvement in analysis times compared to Fig. 5a. (b) Loci of the flow rates corresponding to the analysis times above. Faster flow rates and shorter column lengths are allowed here, compared with Fig. 5 because now fewer plates are lost by extra-column broadening, and therefore, fewer plates are required from the column to accomplish the required separation. (c) Loci of optimal analysis times as a function of the pressure limit for four different fixed column lengths.

limited in this case, and take more time. All of these solutions require 75% methanol, the highest value we allowed in the calculations. For mobile phase compositions below 66% methanol, a 3 cm column produces the fastest analyses (10.66 min at 10 MPa, 5.87 min at 20 MPa, and 4.60 min at 27 MPa and above), but is slower than the best times at 75% methanol.

3.4. Adding particle diameter to the optimization

So far, all our calculations have been done only for the 3.5 μm particles used to collect the model data. It is usually possible to change particle diameter within a stationary phase family and achieve approximately the same overall selectivity if the bonded-phase chemistry is the same. Thus, we can treat particle diameter as another independent variable within our model. We have already seen that some solutions are pressure-limited, and others are plate-number-limited, so increasing the particle diameter would help in one set of instances, and decreasing the particle diameter would help in the other. It is not always clear whether changing the particle diameter will help, or in which direction the change must be made, or how the proper column length, flow rate, and modifier concentration will need to be changed without considering all the other interrelated parameters in concert.

Upon adding particle diameter to the variables, the dimensionality of the model is increased, and it becomes difficult, even for this simple separation, to keep track of all the effects as we have done so far. Fortunately, if we are not particularly interested in understanding all the interactions in actual practice, but only want to find the best conditions to use for our problem, we can simply treat all the changeable variables as continuous, solve for the best combination of parameter values to meet our constraints and minimize time, and then later virtually explore the actual combinations of particle diameter and column dimensions near the optimum to find the best solution from among the available column and particle diameters. It takes only a few minutes of interaction at the computer to reach a final choice of column and corresponding parameter values, but to further illustrate the complexity of the interactions we will show a level of detail here that we normally do not to explore. The optimal analysis times resulting from treating the column length and particle diameter as continuous variables, and using the pressure limit as the independent variable, are given in Table 2.

Surprisingly, we see that the best particle diameter for a time-minimized separation at reasonable pressure is usually larger than 3.5 μm for the current separation when we can freely choose the corresponding column length. However, only 3.5 and 5 μm particles are available in this stationary phase family (Symmetry), and there are only several column lengths regularly available for each particle diameter. We will next consider the best choices from among the available combinations of column and particle dimensions (we will only consider 4.6-mm diameter columns here for simplicity, but if we should need to restrict the flow rate to low values, such as when using mass-spectrometric detection, we would want to add column diameter to the other variables and set a flow rate limit compatible with the detector. Of course, reducing the column diameter will increase the relative peak-broadening contributions from the extra-column volumes). The results of analyzing discrete combinations of column and particle dimensions are given in Table 3 for three pressure limits. Only 5 and 15 cm lengths are available as catalog

Table 2
Optimal analysis times as a function of pressure limit, and the associated flow rates, column lengths, and particle diameters treating the column length and particle diameter as continuous variables[a]

Analysis time (min)	Pressure limit (MPa)	Flow rate (mL/min)	Column length (cm)	Particle diameter (μm)
4.25	5	1.20	3.99	4.58
3.04	10	1.80	4.29	4.12
2.51	15	2.27	4.48	3.86
2.19	20	2.68	4.63	3.69
1.98	25	3.05	4.74	3.56
1.82	30	3.38	4.84	3.46

[a] This is for an HPLC system with 0.020 mL of extra-column volume (see text). The predicted pressure in all solutions was the pressure limit specified. The methanol concentration was constrained not to exceed 75%, and every solution required this concentration.

items in 4.6 mm diameter columns with 5 μm particles, so we added several reasonable custom lengths to the calculations. We included the minimum resolution predicted in each solution because, when variables are changed discretely rather than continuously, resolution is often not a limiting constraint. The critical peak pair was always butylparaben and propranolol.

The fastest analysis in the original experimental data (Fig. 1) took 12.8 min and 18 MPa. Table 3 shows that, even with a 15 MPa pressure limit, there are two solutions that will meet the business needs in analyses requiring less than 4 min. Surprisingly, with a 15 MPa pressure limit, a faster solution is available using a 5 μm packing than when using a 3.5 μm packing.

Raising the pressure limit to 20 MPa reveals a solution requiring less than 3 min. Note that when the pressure limit is raised, any low-pressure solutions that were not pressure-limited at a lower limit still apply with the higher pressure limit. With a 30 MPa limit there is one solution needing less than 2 min. Note that the separation is plate-number-limited if the predicted pressure is below the pressure limit. In these cases the resolution of the critical pair is 2.00 and a somewhat longer column or smaller particles could produce a faster analysis. When the predicted pressure is at the pressure limit the separation is pressure limited, excess resolution is produced, and a somewhat shorter column or larger particle diameter could produce a faster analysis meeting the resolution requirement.

Table 3
Optimal analysis times for discrete particle and column dimensions at three different pressure limits, and the associated flow rates, predicted pressures, and critical-pair resolution (Rs)[a]

Analysis time (min)	Particle diameter (μm)	Column length (cm)	Flow rate (mL/min)	Pressure required (MPa)	Rs
Pressure limit: 15 MPa					
5.16	3.5	3	0.74	3.98	2.00
3.79	3.5	5	1.67	15	2.43
8.53	3.5	7.5	1.12	15	3.53
9.91	5	3	0.39	1.02	2.00
3.46	5	5	1.84	8.07	2.00
4.18	5	7.5	2.28	15	2.44
16.70	5	15	1.14	15	4.37
Pressure limit: 20 MPa					
5.16	3.5	3	0.74	3.98	2.00
2.85	3.5	5	2.23	20	2.27
6.40	3.5	7.5	1.49	20	3.30
9.91	5	3	0.39	1.02	2.00
3.46	5	5	1.84	8.07	2.00
3.13	5	7.5	3.04	20	2.25
5.57	5	10	2.28	20	2.89
12.52	5	15	1.52	20	4.07
Pressure limit: 30 MPa					
5.16	3.5	3	0.74	3.98	2.00
1.90	3.5	5	3.35	30	2.04
4.26	3.5	7.5	2.22	30	2.99
9.91	5	3	0.39	1.02	2.00
3.46	5	5	1.84	8.07	2.00
2.12	5	7.5	4.50	29.64	2.00
3.71	5	10	3.42	30	2.58
8.35	5	15	2.28	30	3.65

[a] This is for an HPLC system with 0.020 mL of extra-column volume. All the calculations were limited to a maximum of 75% methanol, and all the solutions required this concentration. The critical pair was butylparaben and propranolol.

With this information, a worker can now more easily understand the choices available, the tradeoffs between analysis time and pressure, and the influences of other parameters. For the fastest analysis times, a 3.5 μm packing in a 5 cm column is the best combination for this separation. These conditions save about 85% of the time compared to the fastest analysis in the screening data, but 30 MPa pressure is required. If it is important to keep the pressure reasonably low, like in a plant application where HPLC maintenance may be more difficult to manage than in an R&D environment, then the 3.46 min solution requiring only 8.1 MPa with 5 μm packing and 5 cm column is very appealing. This solution still saves 73% of the time required compared to the fastest screening experiment.

3.5. Ultra-high pressure, ultra-small particles

Let us consider the current separation model on an ultra-high-pressure instrument with a 690 MPa (100,000 psi) pressure limit and 0.020 mL of extra-column volume. With a 3.5 μm packing, the best analysis time is 1.45 min on a 4.6 mm × 5.7 cm column, but this would require only 51 MPa if the flow rate is limited to 5 mL/min. Utilizing higher pressure would require increasing the flow rate range or decreasing the column diameter. With a 2.1 mm diameter column, the full range of 690 MPa can be utilized, but the analysis time is essentially unchanged. In this case, the performance limits arise from extra-column volumes. It is pointless to consider using smaller particles without greatly reducing the remaining extra-column volume.

We virtually reduced the extra-column volume in our model to 0.001 mL, the virtual injection volume to 1 μL, and the virtual detector cell volume to 0.5 μL. Analytical columns are usually not available in lengths shorter than 3 cm, so this was set as the minimum column length, and the column diameter was set at 2.1 mm to better match the flow-rate and pressure ranges. We then performed the optimization calculations for our model separation using particle diameters ranging from 1.7 to 5 μm with three different combinations of flow rate and pressure limits: 1 mL/min and 103 MPa (15,000 psi), 2 mL/min and 62 MPa (9000 psi), and 5 mL/min and 41 MPa (6000 psi). Fig. 7 shows the loci of optimal analysis times. The associated parameter values are given in Table 4.

The smallest particles did not produce the fastest analysis times for this problem. So, the performance advantages of ultra-small particles will not result in business benefits in every situation. The fastest analysis time among these results, 0.65 min, calls for 2.5 μm particles at 1.23 mL/min and 62 MPa. This solution, like many in the table, gives excess resolution, and there is no way to exchange this for more speed without changing the constraints. Here the column length is the limiting constraint. It needs to be shortened to go faster in the present separation, and is already at the minimum we

Table 4
Optimal analysis times as a function of particle diameter, and the associated flow rates, predicted pressure, column lengths, and critical-pair resolution for an HPLC with 0.001 mL of extra-column volume[a]

Analysis time (min)	Particle diameter (μm)	Column length (cm)	Flow rate (mL/min)	Pressure required (MPa)	Rs
Other constraints					
1 mL/min, max; 103 MPa, max					
0.84	1.7	3	0.94	103	3.00
0.79	1.8	3	1	97.5	2.85
0.79	2	3	1	79.0	2.66
0.79	2.5	3	1[b]	50.5	2.29
0.79	3	3	1[b]	35.1	2.01
0.97	3.5	3.68	1[b]	31.7	2.00
1.68	5	6.34	1[b]	26.7	2.00
2 mL/min, max; 62 MPa, max					
1.40	1.7	3	0.57	62	3.38
1.25	1.8	3	0.64	62	3.18
1.01	2	3	0.79	62	2.83
0.65	2.5	3	1.23	62	2.16
0.67	3	3.66	1.45	62	2.00
0.82	3.5	4.74	1.52	62	2.00
1.42	5	8.89	1.66	62	2.00
5 mL/min, max; 41 MPa, max					
2.12	1.7	3	0.38	41	3.68
1.89	1.8	3	0.42	41	3.48
1.53	2	3	0.52	41	3.13
0.98	2.5	3	0.81	41	2.42
0.75	3	3.15	1.11	41	2.00
0.91	3.5	4.05	1.18	41	2.00
1.53	5	7.51	1.30	41	2.00

Other constraints are listed in the table.

[a] The critical pair is butylparaben and propranolol in every case. The column diameter is 2.1 mm in every case. The minimum column length was 3 cm. The methanol concentration was constrained not to exceed 75%, and every solution required this concentration.

[b] Note that when the pressure drops below 62 MPa the 1 mL/min flow rate limit is pointless if 2 mL/min is available up to 62 MPa.

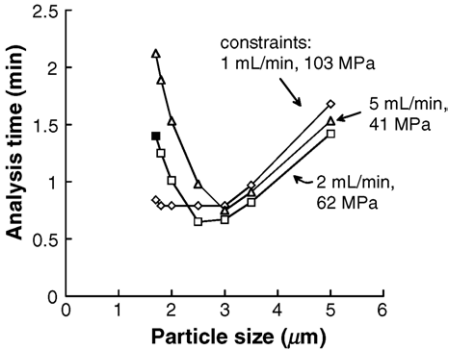

Fig. 7. Loci of optimal analysis times as a function of particle diameter at three different pressure limits. The extra-column volume was virtually reduced to 0.001 mL. The flow rate and column length were allowed to vary as necessary within the specified pressure and flow rate upper limits shown on the figure to produce the optima for each particle diameter. The minimum column length was 3 cm. The values of flow rate, pressure, column length, and the resulting resolution are listed in Table 4. Excess resolution exists on the left side (where the slope is negative) of each curve and in the flat portions, when present. This cannot be exchanged for faster analysis times. The resolution of the critical pair is 2.0 whenever the slope is positive.

allowed. Particles smaller than 2.5 μm require longer analysis times, but provide even more excess resolution for this separation. Here we assumed the packing characteristics of all the particles under consideration would be the same, and that Darcy's law would predict the pressure. We must keep in mind that deviations from this assumption could lead to different pressures and different specific outcomes in an actual instrument, but the trends we predict here are still valuable in understanding how the various parameters interrelate.

An interesting alternative solution for this problem is the 0.91 min analysis at 41 MPa utilizing 3.5 μm particles. The column length for this solution is not available, but this particle diameter would solve the problem in about 1.2 min at only 18 MPa in a 4.6 mm × 3 cm column using a conventional HPLC with minimal extra-column volume and a small-volume detector. A 5 cm column would take more time and more pressure, and would provide excess resolution.

4. Conclusions

When HPLC results are not needed immediately, instruments equipped with autosamplers and running unattended can take as long as necessary with few business consequences. Long sample preparations also reduce the value of HPLC analysis-time savings. However, consider that Soxhlet extractions requiring as many as 24 h were once common for sample preparation. Newer techniques like accelerated solvent extraction are approximately 100-times faster [18]. Even today, the most desirable sample preparation for industrial and commercial applications is simply diluting the sample in an appropriate solvent. Analysis time reductions like those shown here are already very significant and valuable in such situations, particularly if a manufacturing process is being held pending HPLC analysis results.

Experimental optimization is made so complicated by parameter interdependencies that it is not possible to develop the required knowledge in a reasonable time. We determined over 200 optima in developing this paper, and each one would have taken dozens of actual chromatograms to determine with the same precision as our calculations. Numerical modeling and multivariate optimization are extremely valuable for quickly understanding how various parameters and their interactions affect a separation, and what is the best-possible outcome given the business need, the parameters that can be adjusted, and realistic constraints. We showed how to improve a separation from about 13 min to less than 8 min using ordinary columns without making HPLC modifications, and to under 2 min on a system with extra-column volume matched to the column performance.

Plate-number-limited separations can be made faster by using smaller particles and appropriate column lengths because the required plates can be generated quickly if enough pressure is available. But, separations that are not plate-number-limited will take more time than is necessary if the particle diameter is smaller than optimal.

It would be unwise to assume that the separation we examined here is typical or that the conclusions for this separation could be applied to any other. Every problem is different and needs to be treated individually. Fortunately, problems do not need to be studied to the same level of detail as we showed here. Instead, once the data are available for a retention model, the optimum can be determined, the neighborhood of the optimum in its parameter space can be further investigated virtually, and appropriate parameter values can be chosen in only a few minutes.

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