Unexpected Results in Chromatography

Imre Molnar, Institut für angewandte Chromatographie, Berlin, Germany.

Unusual experiments can provide surprisingly good analytical solutions. When developing chromatographic methods, analysts must use a combination of experience and instinct to choose initial starting conditions. This is often followed by a period of trial-and-error optimization until the desired methodology is achieved. This article illustrates how the process of chromatographic method development can be improved using computer modelling and simulation.

Introduction

In general, method development is based on
- personal experience
- available time
- available resources.

   However, personal experience is limited by the professional history of the chromatographer and both time and resources are very often in short supply. To further complicate matters, chromatographic methods often form part of the documentation required to assure the quality of chemical or pharmaceutical products.

   Experienced chromatographers, using their knowledge of conventional methodologies, can often find ways of separating complicated samples. In contrast, non-conventional approaches can sometimes provide answers to complex chromatographic problems; however, these approaches are not intuitive and thus are difficult to conceive. When faced with chromatographic challenges that are not resolved using conventional means, frustration and doubt often set in. What am I doing wrong? What do I do next? Part of the answer may involve computer-supported software tools. This article will show, through a series of examples, how alternative approaches to analytical challenges using software tools can aid in the development of high-quality chromatographic methods.

Experimental

Data in Table 4 were generated on a DX-500 (Dionex Corp., Idstein, Germany) equipped with an EG40 electrolytic eluent generator with EGC(OH) cartridge. The dwell volume was approximately 300 µL. An AS 17 column (Dionex Corp.) of 250 × 2 mm, 10 µm was used. Eluent A was deionized water and eluent B was OH⁻ in water (generated from eluent A by electrolysis). The gradient was 1–60 mM OH⁻ in both 10 and 30 min. The flow-rate was 0.4 mL/min. Method modelling was performed using DryLab software (LC Resources Inc., Walnut Creek, California, USA, or Europe: Molnar, Berlin, Germany).

   Chromatographic conditions for Figures 1 and 2 were as follows: Nucleosil C18, 125 × 4 mm, 5 µm; flow-rate: 1 mL/min; Eluent A: 50 mM phosphate buffer, pH 3.5; Eluent B: ACN; dwell volume: 0.25 mL; linear gradient from 5–70%B; other conditions: see figure legends.

Results and Discussion

Today, the high performance liquid chromatography (HPLC) method-development process is well understood (1). In particular, Snyder's theory of gradient elution helped in the development of modern software tools to enhance the yield of good experimental results (2). Computers have been used since the early days of reversed-phase chromatography (3) to calculate the behaviour of mixtures, especially in life sciences, but these systems, such as the PDP11, were the size of wardrobes. Thankfully, computer technology has advanced, with hardware becoming both smaller and faster, and

Table 1: *Instances of Unexpected Results in HPLC (Collected by HPLC Course Participants).*

Planned experimental change	Expected result	Experimental finding
Increase temperature	Decreased resolution	Increased resolution
Increase %B	Decreased resolution	Increased resolution
Increased gradient time	Increased resolution	Decreased resolution
Increased column length	Pressure too high	Pressure reasonable
	Long analysis time	Reasonable analysis time
Increase pH (for acids)	Longer analysis time	Shorter analysis time
Increase pH for zwitterions	By trial and error	Systematic work more successful
Ion pair chromatography	By trial and error	Systematic work more successful
Change column brand	By trial and error	Systematic work more successful
Increase plate number	Larger particles needed	Smaller particles needed

"method development can be quite complicated."

Table 2: *Necessary Basic Runs to Verify Peak Movements in Computer-Supported HPLC Method Development.*

Sample type	Parameter	Difference	Runs
For unknown mixtures:			
All compounds	Gradient time t_G	30 and 90 min	2
		0–100% acetonitrile	
All compounds	Gradient time t_G	30 and 90 min	2
		0–100% methanol	
All compounds	Temperature	20–40 °C	2
All compounds	Two different t_G	30 and 90 min	2
	Two different temperatures	30 and 70 °C	2
For known mixtures and available but troublesome methods			
Neutrals, acids	%B or t_G	15–20% or 30 and 90 min	2
Basic compounds	%B	10–15% distance	3
Acidic and basic samples	pH	+0.6 and –0.6 units	3
Neutrals	Ternary composition	ACN, MeOH, 1:1-mix	3
Ions	Ion pair agent conc.	factor 0.5 and 2	3
Ions	Buffer conc.	factor 0.5 and 2	3

Figure 1: *Conditions (a) temperature: 60 °C, gradient run time: 40 min. Critical peaks are 1–2, 8–9, 15–17 and 18–19. (b) temperature: 30 °C, gradient run time: 40 min. Critical peaks are 1–2, 11–12, 15–16 and 18–19. The separations of peaks 11–12 and 13–14 are better at the higher temperature of 60 °C.*

software more powerful and user-friendly.

A major fear for chromatographers when applying such computer tools is lack of precision. However, it has been demonstrated that the differences between predicted and experimental retention times can be as little as a few seconds (4).

The following discussion will illustrate some of the surprising findings that can be uncovered from relatively simple experiments using computer simulation and modelling software.

Table 1 highlights some unexpected HPLC results as reported by participants of a series of HPLC courses. Many of these are obviously, and some are surprisingly, untrue. Quite often, changes in resolution occur, but not as we would expect. Most chromatographers have experienced how separations can be improved at higher per cent organic concentration or using a steeper gradient, or how peak pairs can be resolved at higher temperatures. As you'll agree, method development can be quite complicated. So what can we do?

First, let me suggest a series of basic experiments (Table 2). The goal here is to generate a series of chromatograms that are markedly different, by performing a series of runs with large differences in % eluent B, gradient slope, temperature and pH. These basic experiments will show how different starting run conditions affect peak retention times (direction of movement in the chromatogram). If we know how certain parameters affect peak movement we can begin to think about how changing them will improve overall chromatographic resolution. For example, we can alter starting conditions such that resolution of (distance between) critical peaks increases, while the resolution of non-critical (well-resolved) peaks decreases. Ideally, we will create "equal band spaced" peaks. This will, in turn, both increase method robustness and reduce total analysis time.

Once optimized peak distances have been achieved, column length can be reduced whilst maintaining a satisfactory separation. Reducing column length reduces column pressure proportionally, which therefore enables higher flow-rates and faster analysis times. Thus, we have a double time saving, reduced column length and increased flow-rate, without affecting resolution. Software tools can be used to speed up this entire optimization process.

The first example involves the simultaneous optimization of a mixture of 21 components using four basic experimental variables: gradient slopes over 40 and 120 min, and column
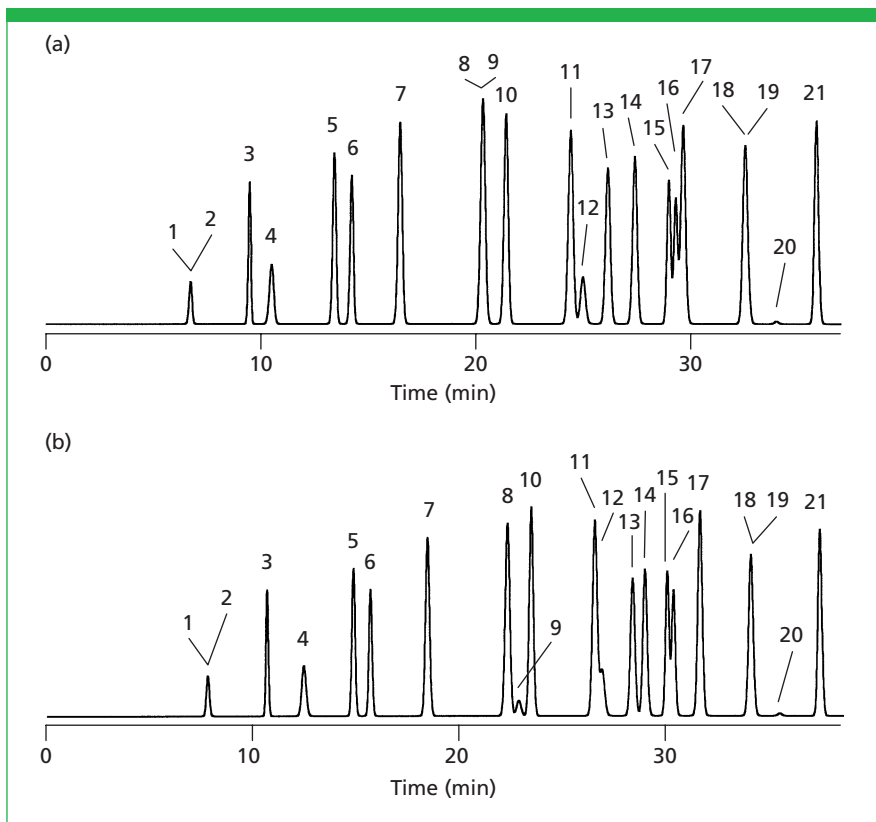
temperatures of 30 and 60 °C (see Figures 1 and 2). As we can see, peak distances vary dramatically. If we are interested in peak 17 it would be wrong to assume that by decreasing the gradient slope (increasing t_G) we would achieve this. A normal approach would be to continue increasing gradient time, and if this was not successful to change the chromatographic column. However, as you can see from Figure 1(b), a satisfactory separation of peak 17 can be achieved at 30 °C using a steeper 40 min gradient run time. Resolution of peaks 11 and 12 also requires an approach that goes against expectations: a higher temperature of 60 °C and a shorter gradient run time of 40 min provide the best resolution. We might have expected the lower temperature (30 °C) to give the better results.

Using the results of the four basic experiments it is possible to build up a three-dimensional resolution map of this separation using computer software (see Figures 3 and 4). These resolution maps can be used to adjust the run conditions depending on the importance of the peaks of interest. Ideally it would be possible to separate all the peaks with a resolution of at least 1.5. However, this is not often the situation, especially if small peaks are present and must be considered. Resolution maps can be altered to consider only the more important peaks.

Another variable to consider in the method development process is the column: we could perform the same separation using a column of increased length with smaller particles and achieve the same average performance. The question to ask in this situation is how will the pressure be affected. If the original column (125 × 4 mm, 3 μm) produces a pressure of 580 psi then we can expect a 250 × 4.6 mm, 3 μm column using the same gradient to generate ≈3300 psi, which is tolerable. Increasing the flow-rate to 1.2 mL/min will increase the pressure still further to ≈3900 psi. The separation is shown in Figure 5.

A step gradient: The next step is to shorten the retention time of peak 1 by increasing the starting %B from 5 to 15% acetonitrile. This reduces the retention time of peak 1 to ≈8 min, a saving of approximately 10 min (Figure 6). Subsequent steps can also be incorporated to bring forward other peaks without affecting their resolution. The next critical peak pair, 4 and 5, require a gradient slope of >1.7%/min for baseline resolution. The second gradient point is then fixed at 10.5 min and 34%B. Table 3 lists the step gradient profile used to
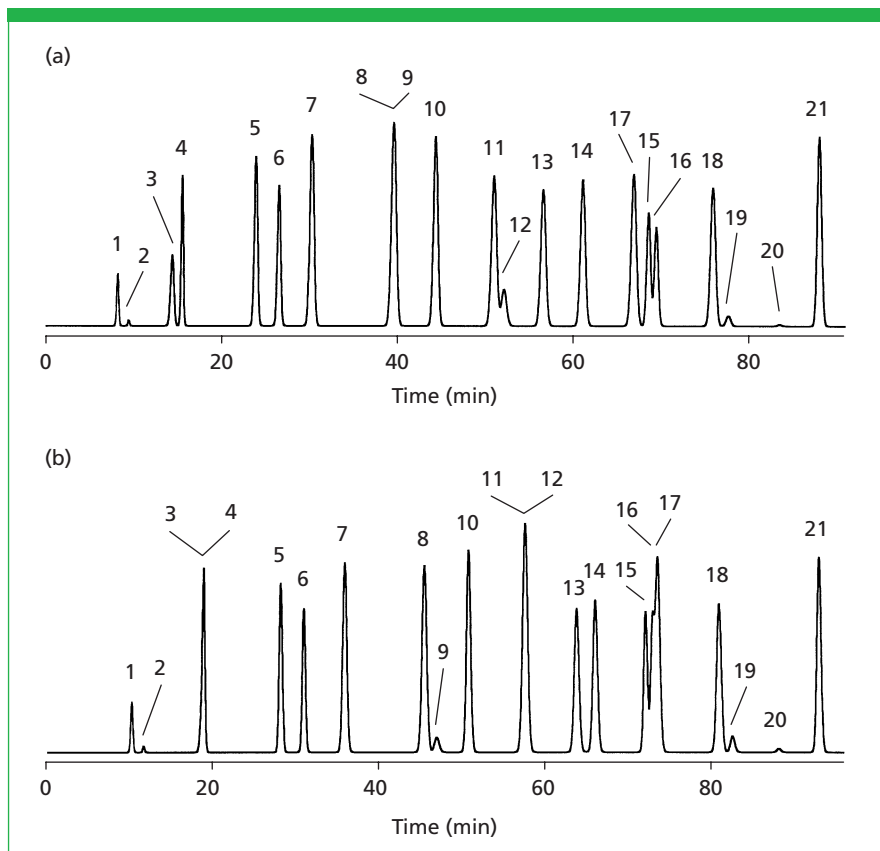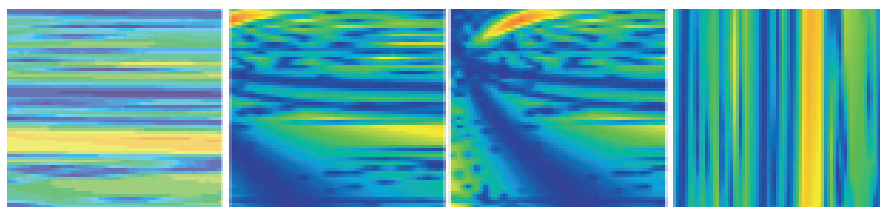

Figure 2: *Conditions (a) temperature: 60 °C, gradient run time: 120 min. Critical peaks are 8–9, 11–12, 15–16. Note the surprising peak position of 17, which is now in front of 15–16. (b) temperature: 30 °C, gradient run time: 120 min. Critical peaks are 3–4, 11–12 and 16–17. The separations of peaks 11–12 and 13–14 are better at the higher temperature of 60 °C. Note the surprising separation of peaks 3 and 4 under the higher temperature. The same is valid for peaks 11 and 12, and 16–17 as well.*
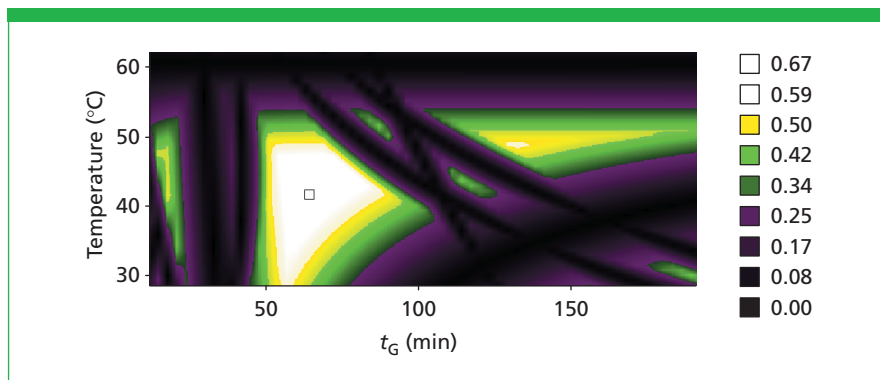

Figure 3: *Critical resolution map of gradient run time (t_G) versus column temperature. The map shows that maximum resolution with the 125 mm, 4.0 mm i.d., 5 μm particle column is only 0.7 at 42 °C using a 65 min gradient run time for peaks 11 and 12. The pressure is 582 psi. The column has 5100 theoretical plates.*

"Because of the nature of analytes in ion chromatography, retention behaviour will be very confusing in complex mixtures."
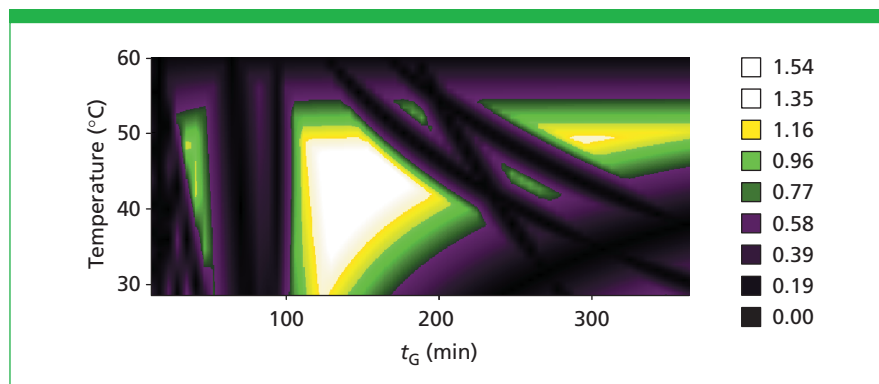

Figure 4: *Critical resolution map of gradient run time (t_G) versus column temperature. The column length has been changed to 250 mm, the i.d. to 4.6 mm and the particle size to 3 µm, while the flow-rate has increased from 1 to 1.2 mL/min. The map shows that there is a large region of R_S >1.5 (white area). The chromatogram at t_G = 140 min and temperature = 42 °C has a critical resolution of 1.5 and also a reasonable pressure of <3000 psi. The flow-rate could be increased to 1.5 mL/min. The critical peak pair is 11–12. Analysis time of the linear gradient (5–70% acetonitrile) is, however, fairly long (>120 min).*
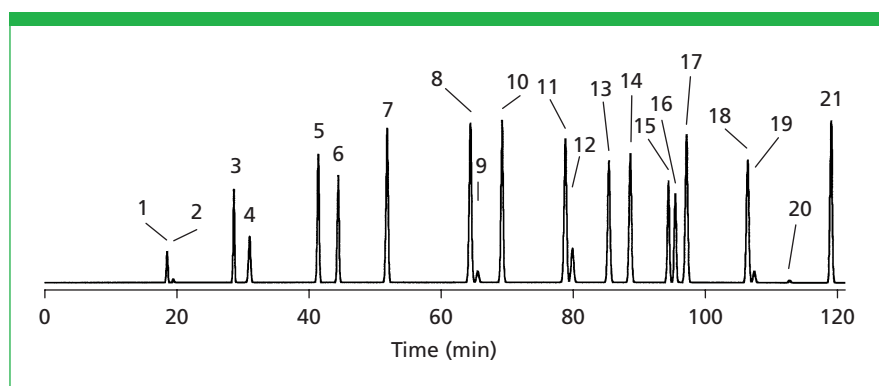

Figure 5: *Linear gradient from 5–70 %B in 140 min at 42 °C, column pressure <3000 psi. Chromatographic conditions as in Figure 4. Although the separation is satisfactory, the analysis takes a long time and should be shortened using step gradients.*
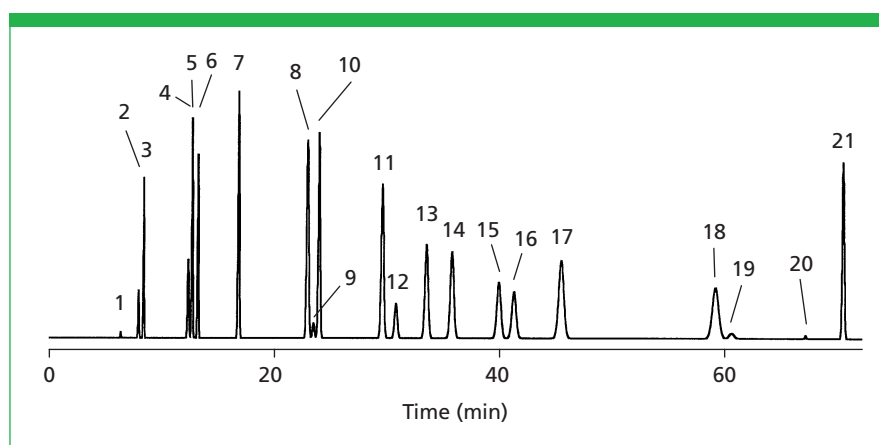

Figure 6: *Optimal step gradients help to reduce analysis time by more than 45%.*

decrease the time of the complete separation. Total analysis time has been reduced by more than 45% (Figure 6).

Unexpected separations in ion chromatography: Retention times can be predicted with high precision in reversed-phase chromatography (4, 9). However, similar studies have not been performed using ion chromatography until recently (11). Ion chromatography gradients can be predicted with extreme precision as shown in Table 4. To generate these data, two basic runs were performed from 0–50 mM NaOH in 10 and 30 min, and a prediction was made by comparing an experimental 20 min gradient with a 20 min computer simulation. In addition, peak areas could also be predicted with good success, as has been described earlier (9). The mean deviation is 5 s, which represents less than 2% error. Because of the nature of analytes in ion chromatography, retention behaviour will be very confusing in complex mixtures. Therefore, computer-supported optimization and control of this method should be applied before the validation has been completed, to avoid unnecessary repetition, in case new peaks are found later on.

Chiral separations: Chiral separations are primarily governed by hydrophobic, ionic and dipole–dipole interactions. For optimizing these separations, it is a good idea to start with two different gradient slopes at two different temperatures. This should provide an excellent insight into the complexity of the mixture, especially if compounds other than the chiral components are present. The optimization strategy is identical to that used in reversed-phase and ion chromatography and has been shown to be greatly enhanced using computer-supported methods (10).

Unexpected choices for column comparison: Column comparison is a

Table 3: *Step Gradient Profile. (See Figure 6.)*

Time (min)	%B	Critical peaks
0.00	15.00	
10.50	34.00	4, 5
14.60	39.00	
18.70	39.00	
23.20	46.00	8, 9, 10
27.00	46.00	
36.16	46.00	
48.80	47.00	
57.80	49.00	
60.40	65.00	18, 19

difficult task, and batch-to-batch reproducibility is often insufficient for compliance with system suitability requirements. In such instances, resolution maps (Figures 3, 4, 7) can offer help when choosing an alternative column. Working conditions for various columns (e.g., temperature and gradient run-time) are chosen such that they appear in the white areas of the resolution map (these areas relate to resolutions of more than 1.5). The result of this is that in the event of column failure a different column from a different manufacturer can be used without the need to revalidate the method.

Unexpected choices in eluent pH adjustments: One surprising influence arises from changes in the pH of eluent A. Of particular importance is the affect of pH on critical resolution, prior to validation. Method

transfer is easier if the relative resolution map is established for pH. Figure 8 shows that if pH studies are not performed in the initial validation, then in almost every instance revalidation is required. In addition, remember to include all degradation products in validation studies from the very beginning or a third adjustment of pH may be necessary.

Two-dimensional pH vs t_G testing: Optimization of gradient slope followed by optimization of eluent A pH provided interesting results. Certain peak groups merged together while others drifted apart. This behaviour could be used to optimize the pH and gradient slope (12). Figure 9 shows an example that contains the data of six runs: three pH values at two gradient run times. The map highlights the choices available for method optimization. The best
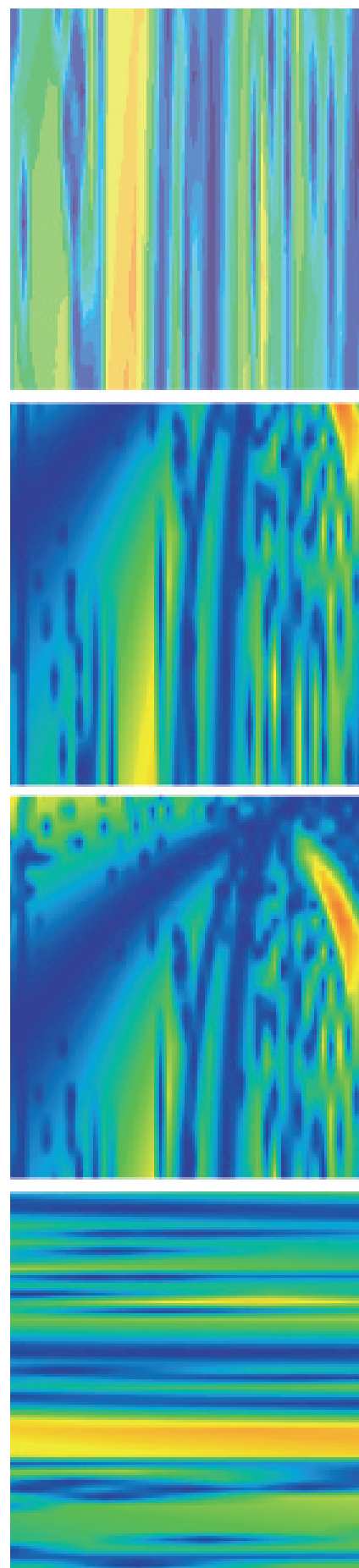
Table 4: *Precision of Predicted and Experimental Retention Times in Ion-Chromatography.*

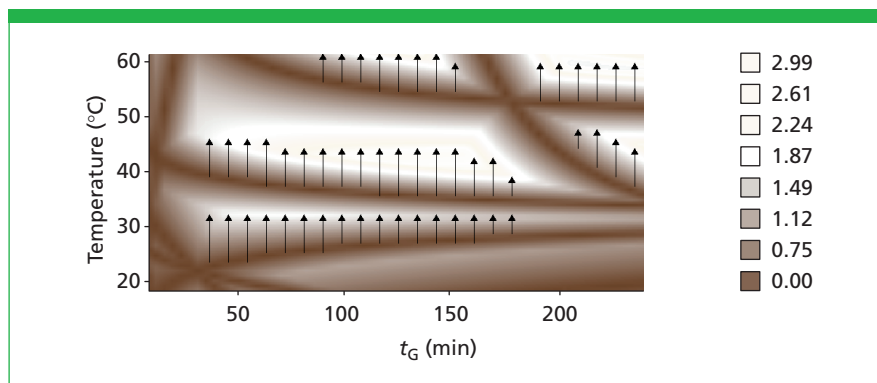| No.	Peak name	t_R	t_{exper}	Diff	Differ (s)
1	Fluoride	2.27	2.25	0.02	1
2	Acetate	2.37	2.38	−0.01	1
3	Formate	2.59	2.57	0.02	1
4	Chloride	3.38	3.32	0.06	4
5	Nitrite	3.64	3.58	0.06	4
6	Bromide	4.50	4.40	0.10	6
7	Nitrate	4.68	4.57	0.11	7
8	Malate	5.38	5.27	0.11	7
9	Tartrate	5.60	5.48	0.12	7
10	Sulfate	5.90	5.78	0.12	7
11	Oxalate	6.23	6.23	0.00	0
12	Phosphate	7.23	7.12	0.11	7
13	Citrate	9.19	9.05	0.14	8
				Average difference	5 s

Figure 7: *A two-dimensional resolution map of temperature and gradient run time showing the critical resolution (scale on right) between $R_S = 0$ (dark) and $R_S = 3.0$ (white). All experiments, characterized by an arrow, start from a dark area (peak overlap) and move in the direction of higher temperatures, (by 5–10 °C), leading to good baseline separation. These experiments are against expectations, even from experienced HPLC experts, and represent unexpected options for resolving critical peak pairs. A large part of the critical resolution map shows such regions, where unexpected improvements are possible (5).*

"Good solutions can be found for HPLC method development using modern tools…"

conditions are found in a relatively small area at pH 4 and a retention time ≈30 min. The dark lines point to a large number of coelutions with changing pH, once again highlighting the sensitivity of the method to mobile phase pH. The importance of pH change increases when performing 'life science' analyses, as most components have acidic, basic or zwitterionic characteristics. The strong pH influence on dissociation equilibria is linked to retention behaviour and thus retention times. For

such analyses, predictions based on molecular parameters are of limited use: the influence of pH, temperature and ionic strength varies too greatly. When working with mixtures of this type it makes no sense to exchange columns for other brands during initial optimization — the first thing to do is map the resolution depending on pH, temperature and gradient shape. Once this work has been finalized then other columns can be considered.

Robust methods in stability testing: It is important to consider all possible components before method development for stability testing starts (6–8). As there are different sources for new components, such as synthesis byproducts, decomposition products, placebo components etc., one should collect them all and develop the method with all of them. The simultaneous use of the two most important parameters is recommended: temperature and gradient slope. Rugged ranges for these two parameters are shown in Figure 10.

In the routine laboratory, validated methods are used over a long period of time. However, at times these methods must be checked; for example, when resolution values are not met because retention time changes have occurred. The normal reaction to such events is to change the column. However, this may improve separation of the critical pairs, but may reduce resolution of other peaks.

For such situations we have developed a new strategy for their rapid solution. This involves increasing the critical resolution, step by step, by optimizing one or two parameters at the same time. In this way, transparent method development reports can be obtained for a better understanding of the role of the critical bands in dependence of %B, pH, temperature and ternary eluent composition, together with column dimensions and particle size of the packing material (Table 5). In the course of this strategy an increased 'equal band spacing' of critical peaks will be observed. The final column optimization helps to increase the speed of analysis. It is not uncommon to see analysis times reduced by 50%, whilst still maintaining high critical resolution values.

Figure 8: *A relative resolution map (RRM) for determining a rugged pH-value. The original pH for the first method validation process, was 4.7. After discovering several coelutions, the pH was changed to 4.4. The dotted line shows the second version of the RRM in the pH range 4.3–4.6. Some months later, however, a new compound was discovered, which was formed in thermal decomposition studies at 40 °C. This compound overlapped with one of the critical peaks, showing a resolution of only 0.9 (steep part of the RRM at pH 4.4). Again the pH was changed, this time to 4.1, where a broad rugged range was available and where the method is now robust and still in use.*
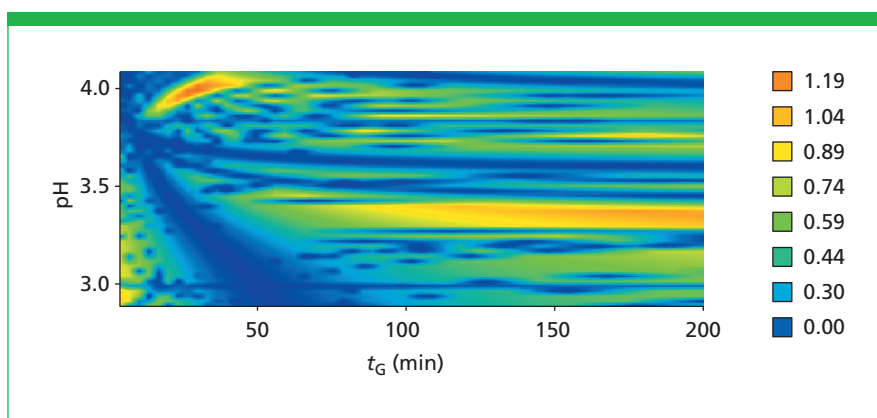
Figure 9: *A three-dimensional plot of critical resolution against pH and gradient run time. The dark areas represent critical R_S = 0 (overlapping peaks). There is a fairly rugged region at pH 3.30–3.45 and t_G between 110 and 200 min, where the analysis time is 40–56 min. Another robust region is at pH 4.0 and 30 min gradient run time. The plot indicates, however, the difficulty in finding the correct pH without having peak overlaps in complex mixtures. Maximum resolution with the 120 mm column is 1.04, so the use of a longer column (250 mm) is recommended. This increases maximum resolution to 1.31. The change in pressure with increasing column length would be from 1200 to 2000 psi.*

Method Transfer

Method transfer is an important issue for validated methods. For gradient methods the role of the dwell volume still requires investigation, but transfer of these methods is increasing and thus new solutions are necessary (1). Computer-assisted development will play a major role
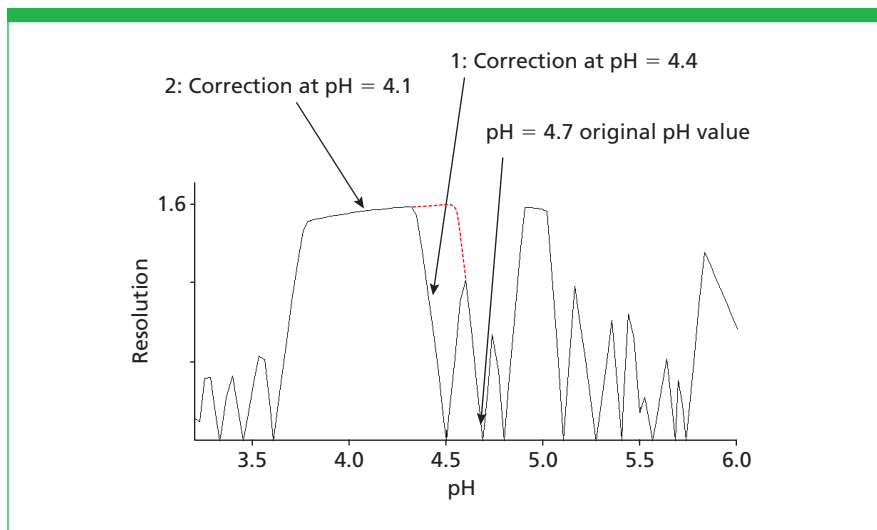
in this work. No method should be transferred without a method development report that documents why the selection of working conditions was the best of all possible choices. Resolution maps can document this properly.

Summary

Good solutions can be found for HPLC method development using modern tools for ruggedness testing, resolution maps and simulated chromatograms. The examples outlined in this article show how computer-assisted method development can provide solutions for difficult separations, which would not otherwise be found.

Acknowledgement

References

(1) L.R.Snyder, J.J.Kirkland and J.L.Glajch, *Practical HPLC Method Development*, (John Wiley and Sons Inc., USA, 1998).
(2) L.R.Snyder in *High Performance Liquid Chromatography. Advances and Perspectives*, Cs. Horváth, Ed., (Academic Press, New York, USA, 1980), 1(4).
(3) Cs. Horváth, W. Melander and I. Molnar, *Anal. Chem.*, **49**, 142 (1977).
(4) R. Dappen and I. Molnar, *J. Chromatogr.*, **592**, 133 (1992).
(5) W. Hancock et al., *J. Chromatogr. A*, **686**, 31 (1994).
(6) I. Molnar, L.R. Snyder and J.W. Dolan, *LC•GC Int.*, **11**(6), 374–387 (1998).
(7) I. Molnar, *LC•GC Int.*, **9**(12), 800–808 (1996).
(8) I. Molnar, *LC•GC Int.*, **10**(1), 32–39 (1997).
(9) I. Molnar, K.H. Gober and B. Christ, *J. Chromatogr.*, **550**, 39 (1991).
(10) M. Lämmerhofer et al., *J. Chromatogr. B*, **689**, 123 (1997).
(11) I. Molnar, Proceedings of the Dionex-Symposium "Ionenanalyse mit Chromatographie und Kapillarelektrophorese," in Idstein, Germany, (September 1999), 277.
(12) H.W. Bilke, I. Molnar and Ch. Gernet, *J. Chromatogr. A*, **729**, 189 (1996).
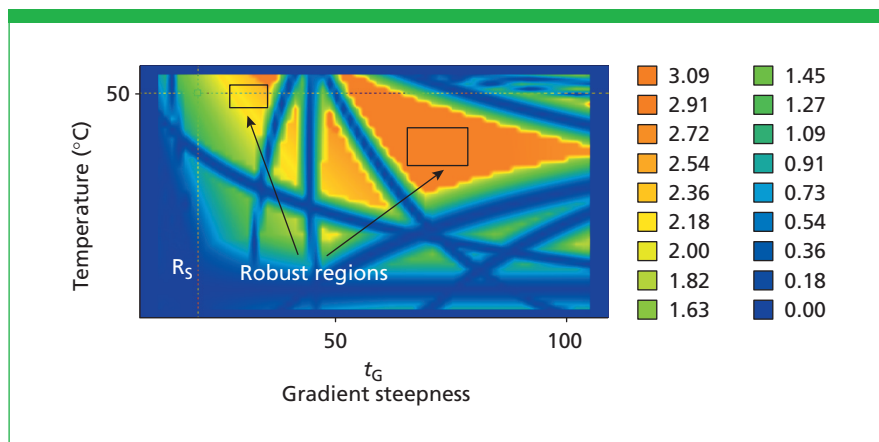(13) H.W. Bilke and I. Molnar, unpublished results.

Figure 10: *A critical resolution map for a "real" pharmaceutical stability sample with 27 components. Robust ranges with critical resolution >1.7 can be established, allowing different selectivities for different critical bands, without changing column and eluent. In this way several variants of the same methods can be applied for different sample components with little additional work. Temperature and gradient changes can be performed without "human interference," which means that several methods can be run for different sample components without equipment "turnaround," tedious column re-equilibration and the set-up of new eluents (13).*
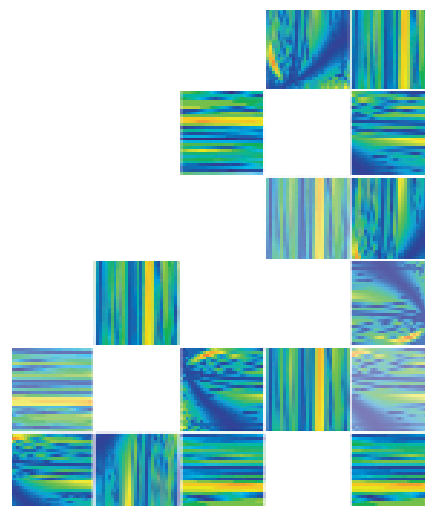
Table 5: *General Strategy in Computer-Supported Method Development and for the Control of Existing Troublesome Methods.*

Step	Action
1	Run two gradients (0–100%B(ACN))(A:0.1 M phosphate pH 2.1) at 30 °C in 40 and 120 min.
2	Repeat the same two gradients at 60 °C.
3	Computer-optimize separation: Look for best °C and best gradient run time. Shape gradient form. This is your "best gradient no.1" at the best temperature.
4	(a) Keeping the gradient form and the temperature constant, now change the pH of eluent A to pH 2.7 (b) Repeat 4(a) but with an eluent A of pH 3.3.
5	Computer-optimize the pH: Look for the highest critical resolution between 1.8<pH<3.6. Run experiment at the pH of the highest critical resolution. This is your "best gradient no.2." Now you have three parameters at their optimum: Gradient form, temperature and pH.
6	Maintaining these conditions, run a further set of experiments: Change eluent B from acetonitrile to methanol, and to a mix of (50:50)(ACN:MeOH)(v:v).
7	Make a resolution map for the ratio of MeOH:ACN, look at the best value and fix the new method at the "best" conditions. This is your "best gradient no.3."
8	In case you still have unresolved peaks, change to another column.
9	Finally the column length, i.d, particle size and the flow-rate can be optimized, considering the allowed and the actual column pressure.