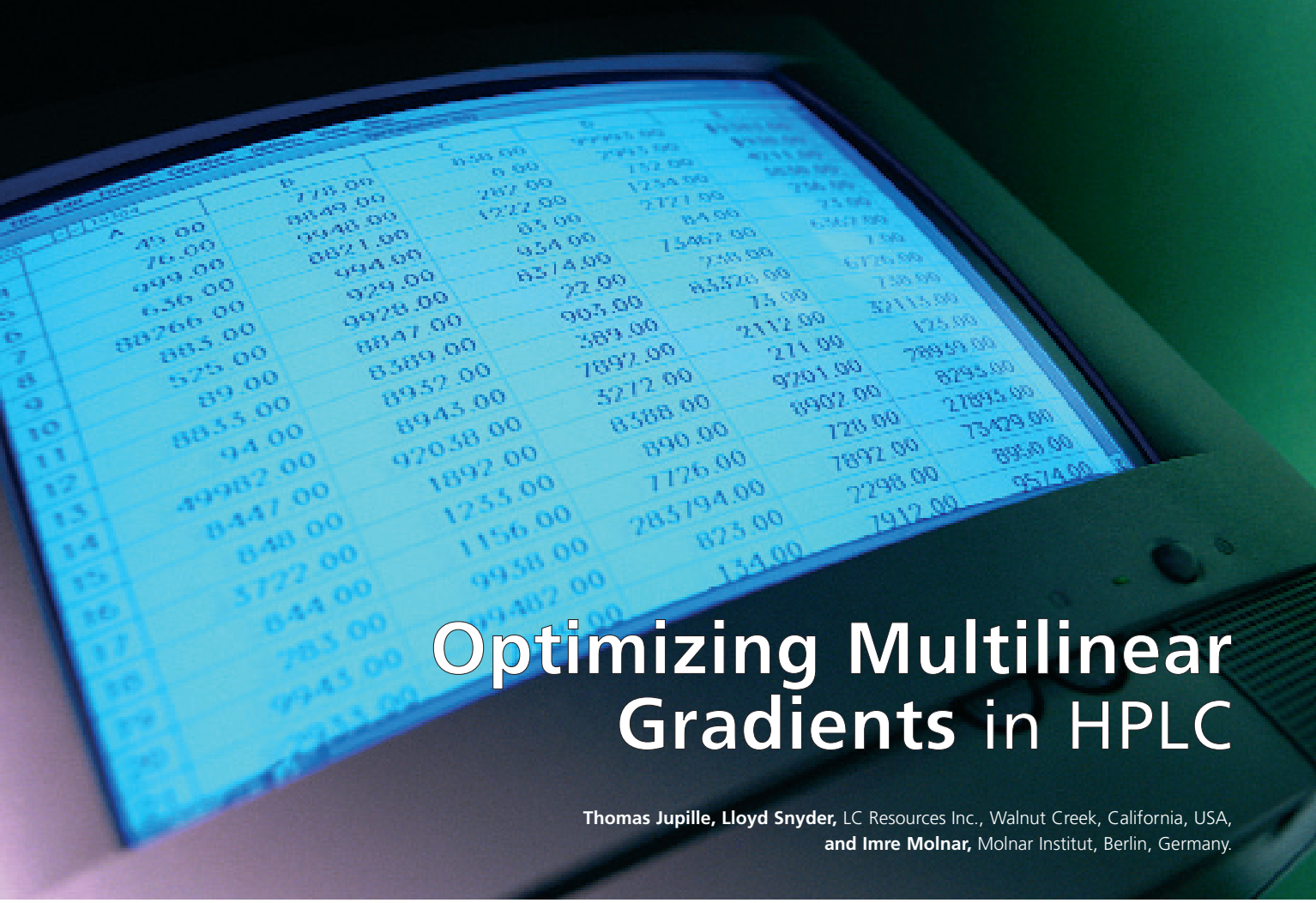


Optimizing Multilinear Gradients in HPLC

Thomas Jupille, Lloyd Snyder, LC Resources Inc., Walnut Creek, California, USA,
and Imre Molnar, Molnar Institut, Berlin, Germany.

Multilinear gradients have not been widely used in general purpose high performance liquid chromatography, in part because of experimental inconvenience in method development. Even with the use of computer modelling, identifying an optimum set of breakpoints has primarily been done by trial and error. Combining a spreadsheet with controllable chromatography modelling software has allowed us to implement a systematic approach to bi- and tri-linear gradient optimization. Application of this approach to 10 randomly generated sample sets suggests that the improvement in critical resolution that can be obtained is considerably smaller than that obtained through the use of a second selectivity variable. Because no additional experimental time (and only limited computational time) is required once an optimum linear gradient has been modelled, however, any improvement thus gained is essentially "free".

Introduction

Non-linear gradients have been around since the beginnings of high performance liquid chromatography (HPLC).[1] When gradients were generated using exponential dilution flasks, curved gradient profiles were arguably easier to generate than linear profiles. With the advent of digital electronics, linear gradients became easier to generate and control. Because curved gradients are extremely difficult to transfer from one instrument to another, when non-linear gradients were desired, they usually took the form of multiple linear segments (we will refer to these as "multilinear" for convenience).

Multilinear gradients have never found much favour for general purpose HPLC applications. Some of the reasons include

- an experiment-intensive optimization process
- transferability issues caused by system-to-system differences in gradient rounding resulting from differences in mixing volume.[2]

The result has been that multilinear gradients are most frequently encountered in dedicated "analysers" (e.g., amino acid analysers) for which the method-development effort can be amortized over a large number of samples and where the analyses will be run on identical hardware platforms.

The major exception has been the use of simple two-segment ("bi-linear") gradients to shorten run time when a separation includes a few well-resolved late-eluting peaks. In this situation, there is sufficient resolution of the late-eluters that the exact breakpoint and slope of the second segment are not critical variables. These separations lend themselves to quick "seat-of-the-pants" (SOTP) development and are not affected seriously by mixing volume differences.

With the development of commercial chromatography modelling software in the late 1980s, SOTP method development could be applied to more challenging multilinear gradients because experiments could be performed on the computer model in a few seconds rather than on the

chromatograph in a few tens of minutes.[3] Useful results could be obtained[3–14] but those results still depended on the chromatographer's experience.

Recently, incorporation of Microsoft Excel 2000 spreadsheets into computer modelling software has facilitated the systematic optimization of bi- and tri-linear gradients. These spreadsheets are available as no-charge add-ons to the core modelling programs and are designed to be easily modifiable by an end-user (who is familiar with Excel macros!) to meet his/her own needs.

The improvement in resolution provided by a multilinear gradient depends on the separation chemistry of the specific sample.

Experimental

Computer modelling was performed using DryLab 2000 Plus (LC Resources Inc., Walnut Creek, California, USA).

The basic algorithm for optimizing bi-linear gradients is simple as shown in Figure 1.

Step 1: Find the optimum linear gradient.

Step 2: Define a grid of possible breakpoints in the gradient profile.

Step 3: Model the separation for all possible breakpoints, keeping track of the critical resolution for each model.

Because steep gradients are notorious for causing modelling and transferability problems,[2,15,17] the model was programmed to not allow instantaneous changes in composition.

In the "standard" version of the spreadsheet, a 19 × 20 grid of possible breakpoints (time/%B pairs) is imposed on the time/concentration range of the optimum linear gradient. Evaluation of the 380 resulting bi-linear gradients takes less than one minute on an "entry-level" PC (950 MHz Intel Celeron processor running Microsoft Windows 2000).

The basic algorithm for optimizing tri-linear gradients is a straightforward variant of the earlier method.

Step 1: Find the optimum linear gradient.

Step 2: Define a grid of possible breakpoints.

Step 3: Model the separation for all possible two-point combinations, keeping track of the critical resolution for each model.

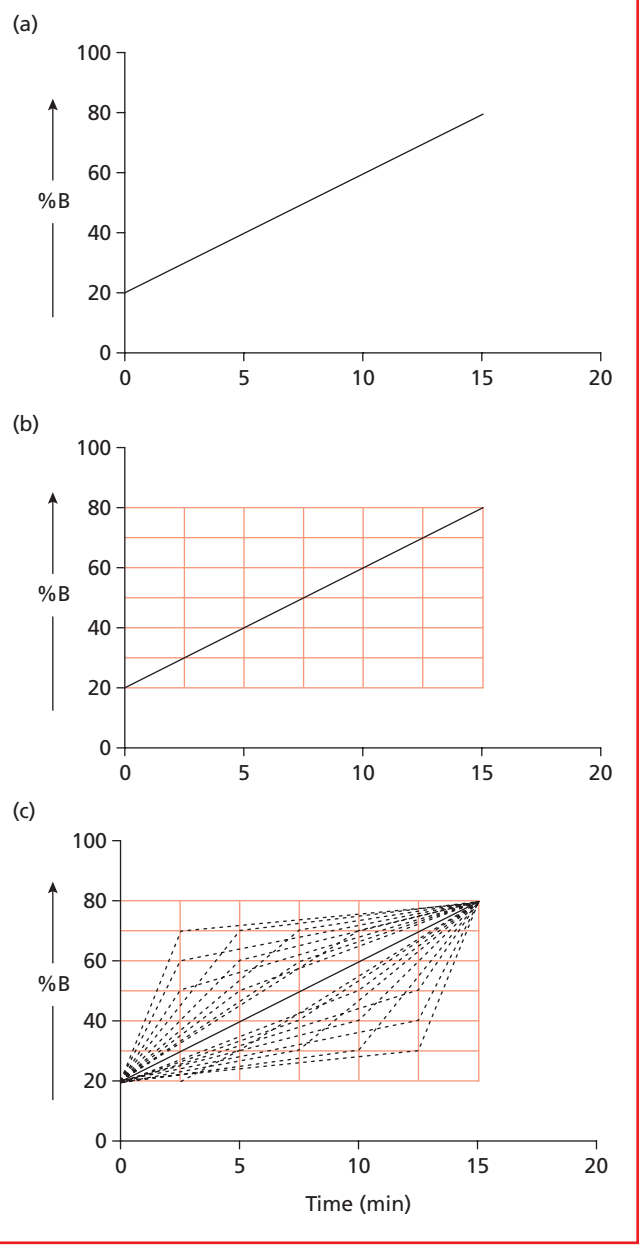
As a matter of practice, the computation time increases approximately as $B^2/2$, where B is the number of breakpoints. Using the same 19 × 20 grid described above would increase the computation time to 2–3 hours. To keep time reasonable, the grid in the tri-linear gradient spreadsheet can be specified by the user. For the results presented here, we standardized on a 10 × 20 grid, which reduces the computation time by a factor of four.

The improvement in resolution provided by a multilinear gradient depends on the separation chemistry of the specific sample. In order to provide a more general evaluation of the capabilities of this approach, we used data for toxicology standards, upon which some results have previously been published.[17–22] These data comprise retention times as a function of gradient time and temperature and were normalized to a consistent set of column dimensions and gradient times. When duplicates were eliminated, we were left with a set of 85 compounds, from which we generated 10 random subsets of 14–18 compounds (Table 1).

Retention data for each subset were entered into both the modelling software and the spreadsheets described above. The following separation conditions (and associated critical resolution) were established for each set:

- optimum linear gradient at 35 °C
- optimum bi-linear gradient at 35 °C
- optimum linear gradient at any temperature between 33–55 °C (and the associated temperature)
- optimum bi-linear modification of 3 (at the optimum temperature found in 3)
- optimum tri-linear modification of 3 (at the optimum

Figure 1: Bi-linear gradient algorithm used. (a) Find the optimum linear gradient; in this hypothetical example, 20–80 %B in 15 min. (b) Impose a grid of possible breakpoints over the optimum linear gradient; a 5 × 6 grid is shown here for clarity; the examples discussed in the text used a 19 × 20 grid. (c) Evaluate critical resolution for each gradient in turn.



temperature found in 3).

Because the optimum gradient conditions depend on desired run time, column dimensions, flow-rate and instrument dwell volume, we ran all simulations under consistent conditions:

- maximum run time = 30 min
- column = 15 × 0.46 mm
- flow = 2 mL/min
- dwell volume = 1 mL.

Results and Discussion

Table 2 shows the critical resolution (resolution between the worst separated pair of peaks) for the optimum linear and bi-linear gradients at 35 °C for the 10 samples. The improvement ranges from 0 (in 4 instances) to 31%. Although the average improvement is 9%, the wide variability suggests that improvement will be substantial in a few instances, but negligible in most.

One question that we wished to answer was how the

Table 1: Ten "random" samples.

Random Sample Number				
1	**2**	**3**	**4**	**5**
Acetominophen	Amphetamine	*N*-acetylprocainamide	Amphetamine	Tripelennamine
Codeine	*N*-acetylprocainamide	Tripelennamine	β-hydroxytheophylline	Methamphetamine
Nitro C3	Acetominophen	β-hydroxytheophylline	Nitro C3	β-hydroxytheophtlline
Ethylmorphine	β-hydroxytheophylline	Methamphetamine	Sulfmethanzine	Nitro C3
Albuterol	Albuterol	Brucine	Brucine	Sulfmethanzine
Clenbuterol	Brucine	Aminoantipyrine	Pyithyldione	Brucine
Norcodeine	Cinchonidine	Ephedrine	Propionylprocainamide	Ephedrine
Nadolol	Propionylprocainamide	Hydrocodone	Nadolol	Pyithyldione
Antipyrine	Nadolol	Pyithyldione	Doxepram	Cinchonidine
Pyrilamine	Hydrocodone	Pyrilamine	indole-3-carboxaldehyde	Chlordiazepoxide
Doxepram	Chlordiazepoxide	Doxepram	Salicylic Acid	Vincamine
Naphazoline	Pyrilamine	Levorphanol	Brompheniramine	Aprobarbital
Butabarbital	Benzoylecgonine	Brompheniramine	Metoprolol	Benzoylecgonine
Butethal	Metoprolol	Aprobarbital	Thebaine	Salicylic acid
Colchicine	Thebaine	Thebaine	Phenacetin	Naphazoline
		Zoxazolamine	Mazindol	Butethal
		Colchicine	Cortisone	Cocaine
			Imipramine	
6	**7**	**8**	**9**	**10**
Metoprolol	Colchicine	Methamphetamine	Tranylcypromine	Tripelennamine
Thebaine	Desipramine	Norcodeine	Methamphetamine	Codeine
Phenacetin	Cortisone	Propionylprocainamide	Cinchonidine	β-hydroxytheophylline
Salicylic acid	Chlorpromazine	Nadolol	Chlordiazepoxide	Brucine
Nitro C9	Diphenhydramine	Chlordiazepoxide	Aprobarbital	Norcodeine
N-acetylprocainamide	Cyclobenazprine	Levorphanol	Pyrilamine	Propionylprocainamide
Halazepam	Tribenzylamine	Naphazoline	Metoprolol	Pyrilamine
Propionylprocainamide	Benzotropine	indole-3-carboxaldehyde	Mazindol	Vincamine
Tripelennamine	Lometazepam	Butethal	Colchicine	Naphazoline
indole-3-carboxaldehyde	Chloroxylenol	Unknown	Imipramine	Salicylic acid
Mefenamic acid	Butylparaben	Mesoridazine	Mesoridazine	Imipramine
Flunitrazepam	Nitro C5	Diphenhydramine	Fluoxymesterone	Unknown
Nitro C5	Danthron	2-Napthoxyacetic acid	Flunitrazepam	Nitro C4
Phenylbutazone	Phenylbutazone	Tribenzylamine	Tribenzylamine	Tribenzylamine
Phentermine	Tamoxifen			Butylparaben
Fluoxymesterone	Biphenyl			Nitro C6
Nitro C7	Nitro C8			
Nitrazepam	Nitro C10			

The use of multilinear gradients can provide a small but significant improvement in resolution for gradient separations where selectivity has not been optimized.

improvement in resolution provided by gradient shape compared with that provided by other selectivity variables. Because we had access to data as a function of gradient conditions and temperature, we compared results for the optimum linear gradient at a single temperature (35 °C) with those for the optimum linear gradient at any temperature within the range of the model (33–55 °C). Results are shown in Table 3. The improvement ranges from 14% to over 200%.

Temperature provides significantly more improvement than does gradient shape, at the cost of only two additional calibration runs. Four experimental runs (two gradients at two temperatures) are required to calibrate the gradient/temperature model versus two experimental runs for the gradient-only model. Once temperature and gradient time have been optimized, a bi-linear gradient provides only slight additional improvement on average (and no improvement for most samples), as shown in Table 4.

Tri-linear gradients are significantly better in this respect, improving resolution by up to 40% (with an average of 8%) as shown in Table 5.

Figure 2 compares the chromatography for the "best-case" sample (no. 8).

We did not explore more complex gradients (although the

Table 2: Comparison of critical resolution for optimum linear gradient and optimum bi-linear gradient at a single temperature.

Critical resolution			
Sample	**Linear 35 ˚C**	**Bi-linear**	**Change (%)**
1, 35 ˚C	0.974	1.277	31
2, 35 ˚C	1.135	1.135	0
3, 35 ˚C	1.215	1.233	1
4, 35 ˚C	0.578	0.627	8
5, 35 ˚C	1.200	1.200	0
6, 35 ˚C	1.702	1.703	0
7, 35 ˚C	0.769	0.798	4
8, 35 ˚C	0.819	0.819	0
9, 35 ˚C	0.763	0.921	21
10, 35 ˚C	0.499	0.596	19
		Avg =	9%
		Std dev =	11%

Table 3: Comparison of critical resolution for optimum linear gradient at a single temperature and optimum linear gradient at any temperature.

Critical resolution			
Sample	**Linear 35 ˚C**	**Linear 33–55 ˚C**	**Change (%)**
1	0.974	1.892	94
2	1.135	1.298	14
3	1.215	1.426	17
4	0.578	1.100	90
5	1.200	1.674	40
6	1.702	2.023	19
7	0.769	1.162	51
8	0.819	1.294	58
9	0.763	2.238	193
10	0.499	1.503	201
		Avg =	78%
		Std dev =	65%

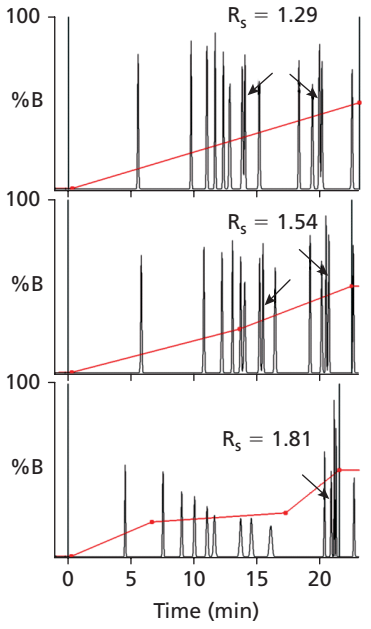
Table 4: Comparison of critical resolution for optimum linear gradient at any temperature and optimum bi-linear variant.

Critical resolution			
Sample	**Linear 33–55 ˚C**	**Bi-linear 33–55 ˚C**	**Change (%)**
1	1.892	1.984	5
2	1.298	1.298	0
3	1.426	1.426	0
4	1.100	1.100	0
5	1.674	1.674	0
6	2.023	2.023	0
7	1.162	1.163	0
8	1.294	1.544	19
9	2.238	2.281	2
10	1.503	1.504	0
		Avg =	3%
		Std dev =	6%

Table 5: Comparison of critical resolution for optimum linear gradient at any temperature and optimum tri-linear variant.

Critical resolution			
Sample	**Linear 33–55 ˚C**	**Tri-linear 33–55 ˚C**	**Change (%)**
1	1.892	2.074	10
2	1.298	1.308	1
3	1.426	1.454	2
4	1.100	1.150	5
5	1.674	1.826	9
6	2.023	2.025	0
7	1.162	1.182	2
8	1.294	1.814	40
9	2.238	2.486	11
10	1.503	1.503	0
		Avg =	8%
		Std dev =	11%

Figure 2: Comparison of optimum linear gradient at any temperature and its bi-linear and tri-linear variants. This was the "best case" (greatest improvement in critical resolution) for multi-linear gradients among the samples studied.



spreadsheet macros are easy to modify if required) because of the potential for method transfer problems caused by instrument-to-instrument differences in mixing volume. For a linear gradient, the resulting errors are limited to early-eluting peaks because mixing volume differences affect the gradient profile primarily at changes of slope.[16, 2] As the number of segments increases, the potential for error also increases.[2]

Conclusions

The use of multilinear gradients can provide a small but significant improvement in resolution for gradient separations in which selectivity has not been optimized. This improvement is "free" in the sense that it requires a short computation time and no additional experimental data beyond that needed to optimize the linear gradient slope.

Multilinear gradients provide less improvement in situations where the separation has already been optimized for gradient steepness and a second selectivity variable (in this instance, temperature). Under these circumstances, bi-linear gradients really provide negligible improvements. Tri-linear gradients can provide a small but significant improvement. Computation time can be significant, but no additional experimental data are required.

The data sets and spreadsheets used can be obtained free of charge by e-mailing the author at tom.jupille@lcresources.com.

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Tom Jupille is Vice President of LC Resources Inc. He has been a practising chromatographer for more than 30 years, during which time he has written more than 30 papers on chromatography and related subjects.

Lloyd Snyder is widely regarded as one of the founders of HPLC and is well known for his 40 plus years of experience and research in the field of liquid chromatography. He has written more than 200 HPLC-related papers and has taught HPLC courses to more than 10 000 students since 1971. He is co-author of the books *Introduction to Modern Liquid Chromatography*, *Practical HPLC Method Development* and *Troubleshooting HPLC Systems*.

Imre Molnar studied chemistry in Saarbrücken in Germany with special training in analytical chemistry and followed up with two years of post-doctoral training with Csaba Horváth at Yale University, USA on basic research about fundamentals of reversed-phase chromatography. He has over 20 years of experience in development of HPLC instruments, columns and HPLC applications.