ELSEVIER

Journal of Chromatography A, 948 (2002) 35–41

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Two-dimensional optimization using different pairs of variables for the reversed-phase high-performance liquid chromatographic separation of a mixture of acidic compounds

T.H. Jupille[a], J.W. Dolan[a], L.R. Snyder[a,*], I. Molnar[b]

[a]*LC Resources, Walnut Creek, CA 94596, USA*

[b]*Molnar Institut, Schneeglockchenstrasse 47, D-10407 Berlin, Germany*

Abstract

Computer-facilitated method development has been extended for the simultaneous optimization of any two variables in separations by HPLC and other chromatographic procedures (gas chromatography, supercritical fluid chromatography, capillary electrophoresis, etc.). The application of this approach to HPLC method development is illustrated by the reversed-phase separation of a nine-component mixture of organic acids. Two of four variables (temperature, solvent strength (%B), pH and buffer concentration) were separately optimized in terms of selectivity, and the results are compared in terms of which variables and other conditions are most effective in providing maximum resolution for samples that contain ionizable compounds.

Keywords: Optimization; Computer simulation; Method development; Resolution; Organic acids

1. Introduction

The importance of HPLC as an analytical technique has led to a considerable literature on preferred ways to carry out method development [1]. The main challenge in optimizing separation is the choice of conditions for maximum sample resolution, and this is largely determined by system selectivity (values of the separation factor α). Since the late 1970s, there has been increasing use of computers as an aid to HPLC method development [1–7], with primary emphasis on the selection of conditions for optimal selectivity or band spacing.

Commercial software which facilitates the optimization of selectivity and resolution has been described and compared [1,7,8]. Until recently [9], available optimization software has restricted the user to a limited choice of separation conditions that can be varied during computer-facilitated method development. A few computer programs allow the user to optimize more than one condition, but only one at a time, while other programs allow the simultaneous change of two variables of predetermined type; e.g., the composition of constant-solvent-strength mobile phases composed of water plus three organic solvents [3]. Ideally, the user would have complete freedom to choose any two conditions for simultaneous optimization, since different pairs of conditions will generally be better suited for different samples, and it is well known that varying two conditions simultaneously can be more effective than optimizing a single condition.

In the present study, selectivity and resolution were optimized for a representative sample of ionizable compounds, using recently introduced software [9] that can predict separation when any two con-

*Corresponding author.

0021-9673/02/$ – see front matter
PII: S0021-9673(01)01587-4

ditions are varied simultaneously. Different pairs of conditions were compared, based on combinations of four variables: temperature, mobile phase (%B), pH or buffer concentration. Some preliminary generalizations from this study appear useful for the similar separation of other samples that contain acidic or basic compounds.

2. Experimental

Retention measurements used in the present study have been reported [10], along with details concerning equipment, sample, materials and procedures. The sample is described in Table 1, which includes approximate pK_a values (determined in the present chromatographic system) for these individual acids. Data from Ref. [10] have been reported for 50 different runs involving different combinations of conditions: temperature, 30, 35 and 40 °C; percent methanol in buffer, 35, 40 and 45%; pH, 2.6, 2.9 and 3.2; buffer concentration, 10, 25 and 40 m*M*. Other conditions are: column, 25×0.46 cm Zorbax C_8 (Agilent); mobile phase, methanol–sodium acetate buffer; flow-rate, 1.0 mL/min.

DryLab 2000 software (LC Resources) was used for these optimization studies. Experimental retention data were entered for four to nine separations, depending on the choice of conditions to be optimized (Fig. 1). A plate number of $N = 18\,000$ was assumed in the various computer simulations of separation.

Table 1
Acidity constants for compounds in the substituted benzoic acid sample. Data from Ref. [11]

Compound	pK_a
(1) 2-Nitrobenzoic acid	2.7
(2) Phthalic acid	3.2
(3) Impurity	3.4
(4) 2-Fluorobenzoic acid	3.6
(5) 3-Cyanobenzoic acid	3.5
(6) 2-Chlorobenzoic acid	3.2
(7) 3-Nitrobenzoic acid	3.4
(8) 3-Fluorobenzoic acid	3.8
(9) 2,6-Dimethylbenzoic acid	3.5
Average	3.4

3. Results and discussion

3.1. General plan

Computer programs for optimizing resolution and selectivity usually require an experimental design; i.e., some defined number of experiments in which the conditions to be optimized are varied. Fig. 1 illustrates the various experimental designs used in the present study. A minimum number of changes in conditions are required for each variable: two for temperature, two for %B, three for buffer, and three for pH. The use of pH as a variable requires measurements differing by no more than 0.5–1.0 pH units [9,11]. When simultaneously optimizing pH and one other condition, as many as eight runs with pH varying can be entered into the program, allowing exploration of pH over a range of 4–8 units.

For each of the experimental designs of Fig. 1, two other (''secondary'') conditions are fixed. This is illustrated in the example of Fig. 2, for an optimization where %B and buffer concentration (''primary'' conditions) are optimized. The secondary conditions of temperature (35 °C) and pH (2.9) were held constant in Fig. 2. The experimental design for the optimization of Fig. 2 (Fig. 1d) requires six input runs. Optimizations as in Fig. 2 were carried out for a wide range in secondary conditions for each pair of variables to be optimized, as summarized in Table 2. A total of 22 optimizations are represented in Table 2, based on the 50 different experimental runs used as input to the computer program.

In the example of Fig. 2, the resolution map indicates a maximum possible resolution of $R_s = 1.4$, for primary conditions of 36 %B and 24 m*M* buffer (note that the R_s scale is approximate, showing $R_s = 1.34$, whereas the actual maximum resolution reported by the software is $R_s = 1.38$). Similar experiments as in Fig. 2 were carried out for all possible combinations of primary and secondary variables, as a means of mapping the resolution of this sample as a function of all four conditions. Resulting maximum resolution values are shown for each optimization in the last column of Table 2.

3.2. Preferred primary and secondary conditions for two-dimensional optimization

Fig. 3 shows an example of the optimization of

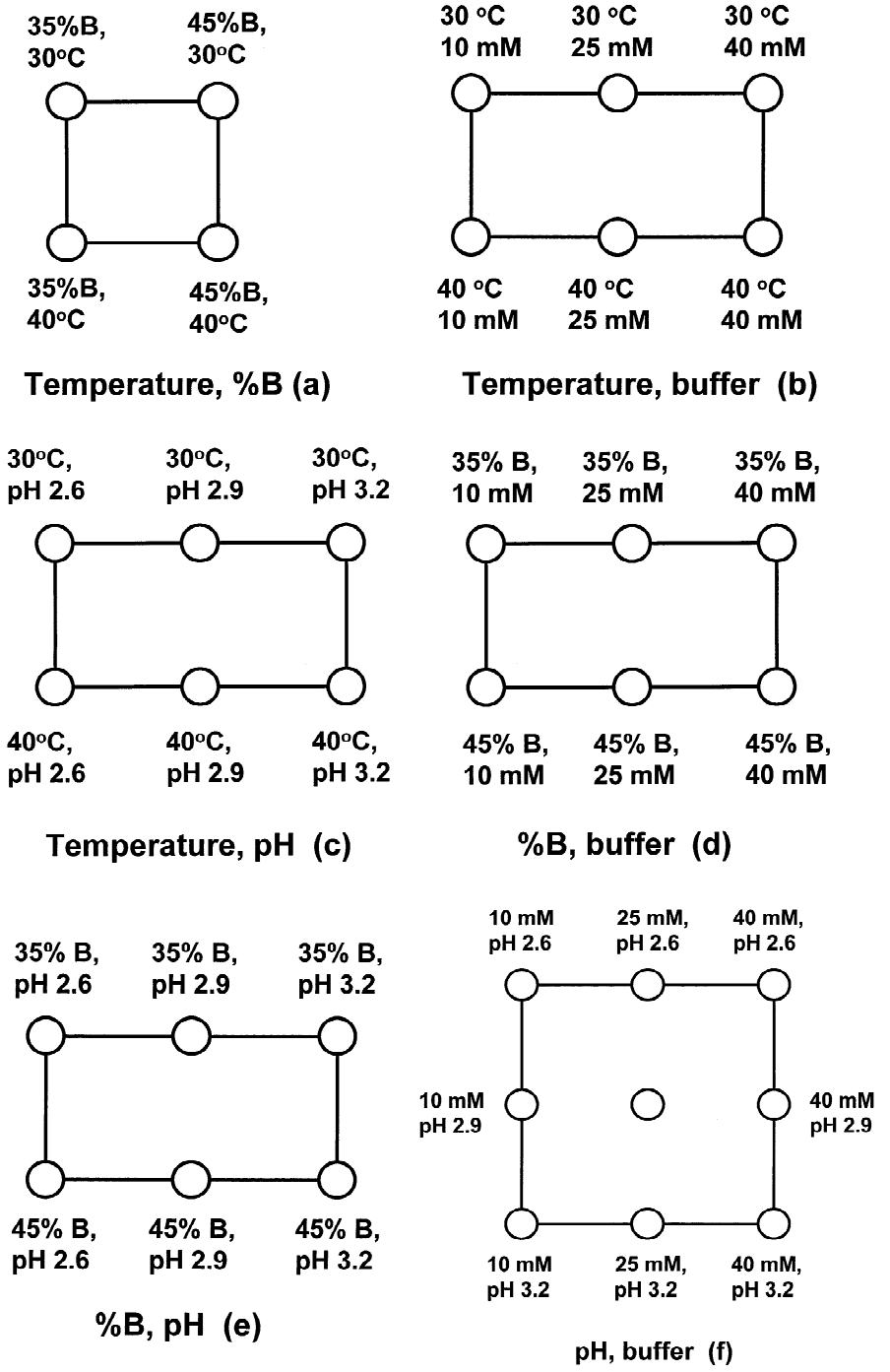

Fig. 1. Experimental designs for two-dimensional optimization based on different combinations of primary conditions.

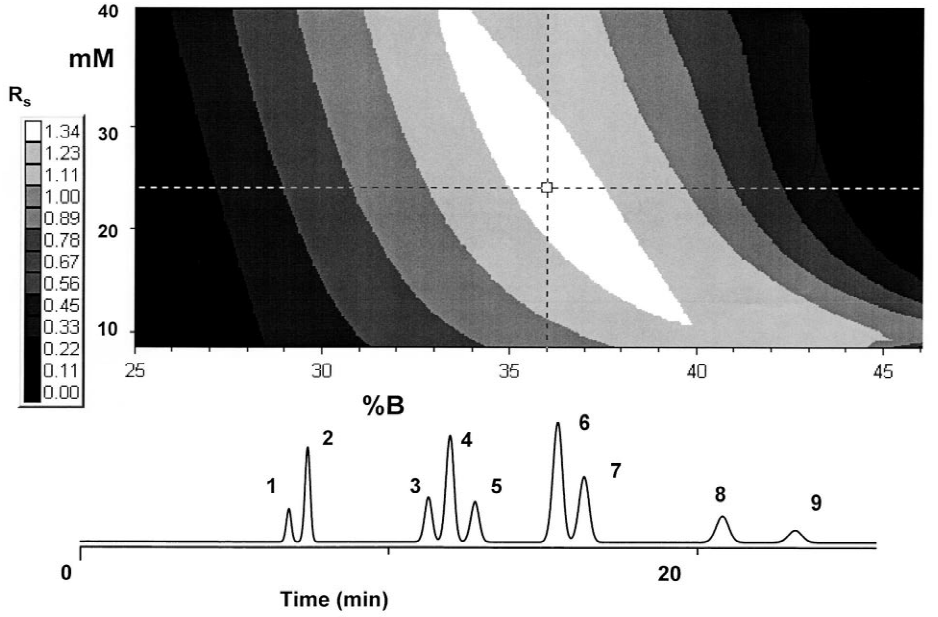

Fig. 2. Optimization of buffer concentration and %B (secondary conditions: 35 °C, pH 2.9). Resolution map and optimized separation (24 m*M* buffer, 36 %B; $R_s = 1.4$). See Table 1 for peak numbering; cross-hairs mark maximum R_s.

temperature and %B, in this case with secondary conditions of pH 2.9 and buffer concentration 10 m*M*. Primary conditions for maximum resolution ($R_s = 1.8$) are 38 °C and 39 %B. Repeating this optimization for other secondary conditions yielded the results of Table 3. To summarize, depending on the starting secondary conditions (pH, buffer), the maximum attainable resolution varied from 0.4 to 1.9. However, four out of these five optimizations gave a final resolution of $R_s > 1.5$ (baseline resolution).

The relative effectiveness of different pairs of primary conditions for achieving maximum resolution for this sample can be evaluated from the data of Table 2. Ideally, the optimization of the primary variable will be relatively independent of the choice of secondary variables, as well as providing acceptable final resolution (e.g., resolution with $R_s > 2.0$ is preferred [1]). This is the case for %B and pH as primary conditions ($2.0 \leq R_s \leq 2.4$), but not for other choices of the primary variables.

We can rank the effectiveness of the individual primary conditions in two ways: (a) by averaging values of maximum resolution (R_s[max]) for all experiments in Table 1 that include a given primary variable and (b) by determining the fraction of optimizations with a successful outcome ($R_s \geq 2.0$). Results from these two evaluations are summarized in Table 4 (''all optimization'' columns); these data suggest that the ability of a primary variable to control band spacing and maximize resolution increases in the order: buffer concentration (less effective)<temperature<%B<pH (more effective). Our experience (unpublished studies) suggests that this ranking of primary variables is applicable to other samples as well.

3.2.1. pH as secondary variable

The two optimizations in Table 2 with pH 2.6 (secondary variable) gave much lower values of maximum R_s (average $R_s = 0.3$). This observation is in fact consistent with other studies. Thus, changes in temperature, %B or ionic strength appear to affect selectivity for ionizable compounds (in part) by creating minor changes in the ''effective'' or apparent pH of the mobile phase, meaning that changes in temperature, %B or buffer concentration can cause similar changes in selectivity as would a change in

Table 2
Optimization experiments and results

"Primary" variables[a]		"Secondary" variables[b]				Max R_s
		T (°C)	%B	pH	Buffer (mM)	
T	%B			2.6	25	0.5
				2.9	10	1.8
				2.9	25	2.1
				2.9	40	1.8
				3.2	25	1.8
T	Buffer		35	2.9		1.9
			40	2.9		1.9
			45	2.9		1.7
T	pH		35		25	2.2
			40		25	1.9
			45		25	1.1
%B	Buffer	35		2.6		0
		35		2.9		1.8
		35		3.2		1.8
%B	pH	30			25	2.4
		35			10	2.1
		35			25	2.2
		35			40	2.2
		40			25	2
pH	Buffer	35	35			2.2
		35	40			1.9
		35	45			1.6

[a] Conditions to be optimized. T = temperature.
[b] Fixed values for other conditions.

pH. This in turn means that when pH does not affect selectivity much [i.e., for pH values much lower than the average pK_a value of the sample (cf. Table 1)], the effects of temperature, %B and buffer concentration on selectivity will be reduced for ionizable compounds. Two previous reports support this hypothesis. First, the separation of an aniline sample [12] gave one-dimensional resolution maps that were almost identical, when resolution was plotted vs. either pH or %B. Second, we have recently found [13,14] for a mixture of 22 carboxylic acids, anilines, pyridines and strong bases that selectivity changes due either to temperature or buffer concentration correlate highly with pH. The ability of temperature, %B and buffer concentration to control selectivity for ionizable samples should therefore increase when the mobile phase pH brackets the pK_a values of the sample components, just as the effects of a change in pH are largest when mobile phase pH ≈ sample pK_a.

3.2.2. Four-dimensional local optimization

Another view of the results of Table 2 is provided by resolution maps for each pair of primary conditions, as shown in Figs. 2–4. For each pair of primary conditions, the resolution maps look generally similar as secondary conditions are changed slightly (resolution maps not shown)— although different values of maximum resolution result. Generally favored conditions can be seen in these maps of ≈30 °C, 35–40 %B, 10–20 mM, and a pH of 2.9–3.1. A simultaneous local optimization of all four conditions [15] gave maximum resolution of this sample for 32 °C, 37 %B, 25 mM and pH 2.9, in approximate agreement with the latter composite values from the maps of Figs. 2–4.

4. Conclusions

The separation of a model mixture of substituted benzoic acids has been studied as a function of four different separation conditions (temperature, %B, buffer concentration and pH) that affect selectivity and sample resolution. Recently available commercial software (DryLab 2000) was used for the simultaneous optimization of different pairs of separation conditions (primary variables), with fixed values for the two remaining conditions (secondary variables) in each optimization. The best results were obtained for the simultaneous optimization of %B and pH; regardless of the values of buffer concentration and temperature (secondary conditions), a maximum sample resolution of $R_s \geq 2.0$ was obtained in each case. Optimization using other pairs of conditions was generally less satisfactory. The ability of different primary variables to control band spacing and maximize resolution increased in the order: buffer concentration (less effective) < temperature < %B < pH (more effective).

When pH was not varied as a primary condition, values of pH (secondary variable) close to the average pK_a of the sample appear to promote the ability of other conditions (temperature, %B, buffer concentration) to control band spacing and maximize resolution—presumably by altering the effective mobile phase pH. For maximum control over band spacing and resolution, the secondary conditions should include a pH value that is close to the average

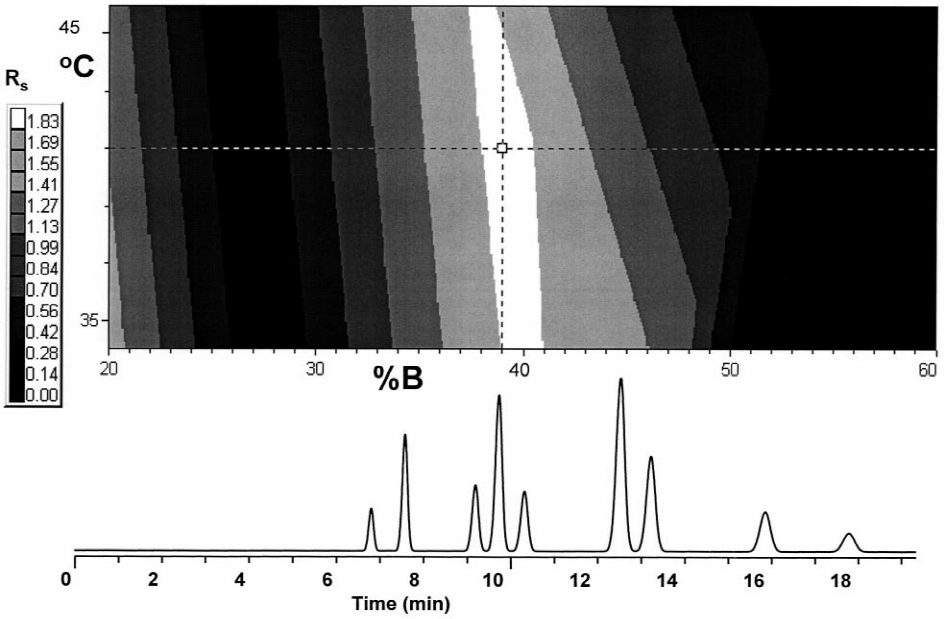

Fig. 3. Optimization of temperature and %B (secondary conditions:10 m*M*, pH 2.9). Resolution map and optimized separation (38 °C, 39 %B; $R_s = 1.9$). Same separation order as in Fig. 2; cross-hairs mark maximum R_s.

pK_a of the sample (i.e., when pH is not a primary variable). When values of pH as secondary variable bracket the pK_a range of the sample, any choice of primary variables can prove effective. Thus, only one of the series of experiments outlined in Fig. 1 may be required for a promising start (or finish) to method development.

The present study involves only a single sample, so some of our conclusions must be considered tentative. The experience of individual chromatographers with their own samples will often suggest a preferred choice of two conditions for simultaneous optimization which may differ from that found for the benzoic acid sample. With the aid of available computer software, method development based on the simultaneous optimization of any two separation conditions can now be carried out efficiently and conveniently, with no limits on the choice of conditions to be varied.

Table 3
Details of optimization experiments for temperature and %B as primary variables

Optimized primary conditions		Secondary conditions		Max R_s
T	%B	pH	buffer	
41	25	2.6	25	0.5
38	39	2.9	10	1.8
35	36	2.9	25	2.1
33	39	2.9	40	1.8
40	32	3.2	25	1.8

Table 4
Relative value of different primary conditions in controlling selectivity and maximizing resolution. See text for details

	Average maximum R_s	% Success ($R_s \geq 2.0$)
Primary condition (1)		
Buffer	1.6	11
T	1.7	18
%B	1.8	46
pH	2.0	64
Primary conditions (2)		
%B, buffer concentration	1.2	0
T, buffer concentration	1.8	0
T, %B	1.6	20
T, pH	1.7	33
pH, buffer concentration	1.9	33
%B, pH	2.2	100

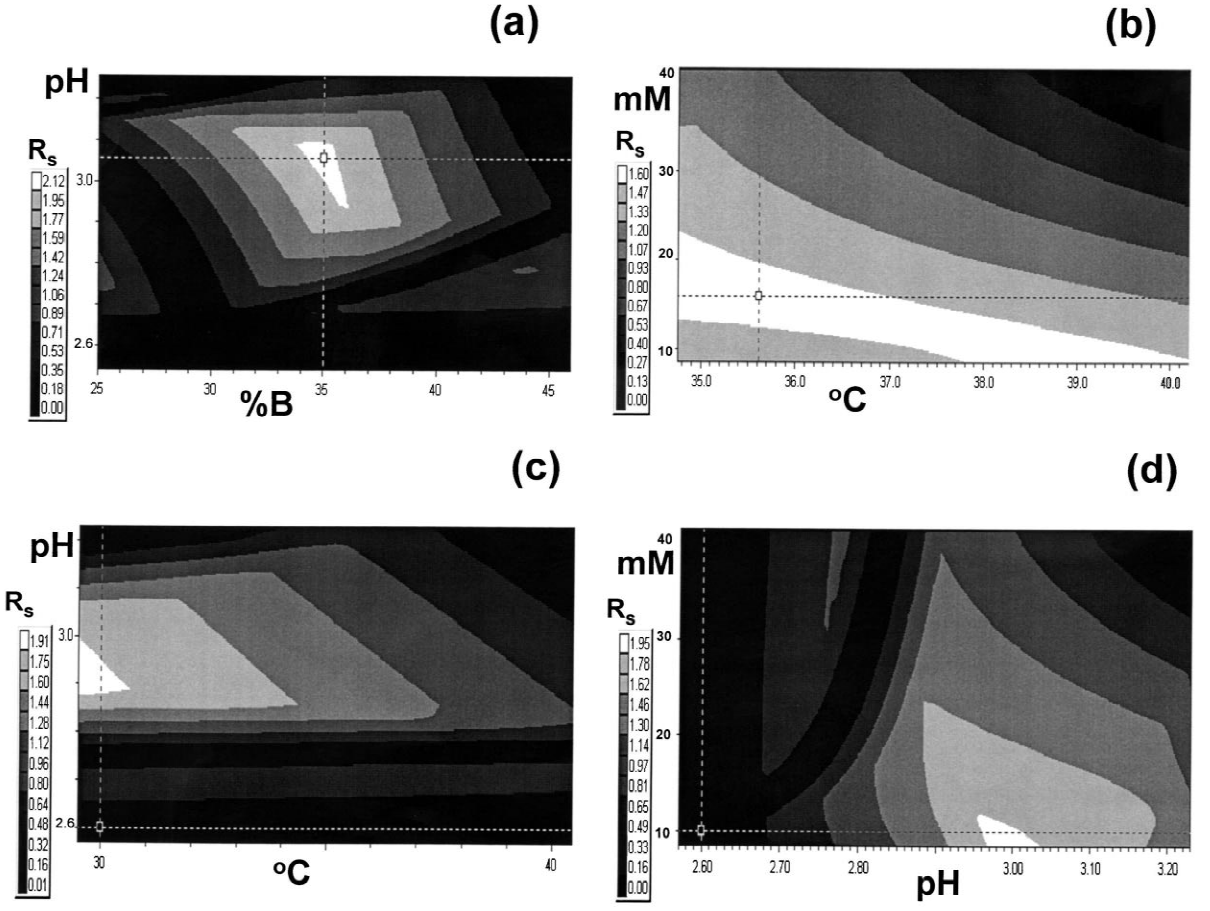

Fig. 4. Resolution maps for different pairs of primary conditions. (a) pH vs. %B (secondary conditions: 35 °C, 25 m*M*); (b) buffer concentration vs. temperature (40 %B, pH 2.9); (c) pH vs. temperature (40 %B, 25 m*M*); (d) buffer concentration vs. pH (35 °C, 40 %B). Cross-hairs mark maximum R_s.

References

[1] L.R. Snyder, J.J. Kirkland, J.L. Glajch, Practical HPLC Method Development, 2nd ed., Wiley–Interscience, New York, 1997.
[2] R.J. Laub, J.H. Purnell, J. Chromatogr. 161 (1978) 49.
[3] J.L. Glajch, J.J. Kirkland, K.M. Squire, J.M. Minor, J. Chromatogr. 199 (1980) 57.
[4] B. Sachok, R.C. Kong, S.N. Deming, J. Chromatogr. 199 (1980) 317.
[5] J.C. Berridge, Techniques for the Automated Optimization of HPLC Separations, Wiley, New York, 1985.
[6] P.J. Schoenmakers, Optimization of Chromatographic Selectivity, Elsevier, Amsterdam, 1986.
[7] J.L. Glajch, L.R. Snyder (Eds.), Computer-Assisted Method Development for High-Performance Liquid Chromatography, Elsevier, Amsterdam, 1990.
[8] P.J. Schoenmakers, J.W. Dolan, L.R. Snyder, A. Poile, A. Drouen, LC–GC 9 (1991) 714.
[9] P. Haber, T. Baczek, R. Kaliszan, L.R. Snyder, J.W. Dolan, C.T. Wehr, J. Chromatogr. Sci. 38 (2000) 386.
[10] J.W. Dolan, D.C. Lommen, L.R. Snyder, J. Chromatogr. 535 (1990) 55.
[11] J.A. Lewis, D.C. Lommen, W.D. Raddatz, J.W. Dolan, L.R. Snyder, I. Molnar, J. Chromatogr. 592 (1992) 183.
[12] J.A. Lewis, J.W. Dolan, L.R. Snyder, I. Molnar, J. Chromatogr. 592 (1992) 197.
[13] N.S. Wilson, M.D. Nelson, J.W. Dolan, L.R. Snyder, P.W. Carr, J. Chromatogr. A (manuscript in preparation).
[14] N.S. Wilson, J.W. Dolan, L.R. Snyder, P.W. Carr, L.C. Sander, J. Chromatogr. A (manuscript in preparation).
[15] L.R. Snyder, J.W. Dolan, D.C. Lommen, J. Chromatogr. 535 (1990) 75.