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Initial experiments in high-performance liquid chromatographic method development
II. Recommended approach and conditions for isocratic separation[1]

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Abstract

Initial method development experiments for both neutral and ionic samples are best carried out with reversed-phase high-performance liquid chromatography (HPLC) using acetonitrile–methanol–buffer mobile phases. The preceding paper (Part I) suggests the use of an acetonitrile-buffer gradient to start method development. In this paper, optimum conditions for this first separation are discussed. Sequential method development experiments plus computer simulations are then used to obtain a final HPLC method. In this connection, we have examined how many experiments are required for reliable predictions of ternary-solvent retention. Three experiments are sufficient to predict isoeluotropic retention for methanol–acetonitrile–buffer ternaries where solvent strength does not vary, but five experiments are required for ternaries that contain tetrahydrofuran.

Keywords: Method development; Isocratic elution; Mobile-phase composition; Nitroxylenes; Nitrotoluenes; Chloroanilines; Ethylanilines; Toluene; Benzyl cyanide; *p*-Cresol

1. Introduction

A number of approaches for reversed-phase HPLC method development have been reported for application to typical samples; i.e., non-enantiomeric compounds with molecular masses <1000 u. The first step is to select conditions for adequate retention ($0.5 < k < 20$) as discussed in the preceding paper [1]. If needed, separation is then improved further by changes in selectivity (α). Several efficient procedures for this purpose have been described, most of which involve retention mapping with the help of a computer [2–6]. For non-ionic samples, constant-strength mobile-phase mixtures of water, methanol (MeOH), acetonitrile (ACN), and tetrahydrofuran (THF) have been used to vary selectivity [7]. Alternatively, solvent strength (%B) can be varied as a means of changing α, using either binary- [8] or ternary-solvent [9] mobile phases. Method development for ionic samples has previously emphasized the use of pH and/or ion-pairing as variables for controlling selectivity [10,11].

The selection of a particular variable or vari-

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[1] For part I see Ref. [1].

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ables (e.g., %B, solvent type, or pH) for the optimization of selectivity and the design of a method development strategy should take into account the factors summarized in Table 1. In the past, more weight has been given to factors 2, 3 and 5 of Table 1 than the remaining factors 1, 4, and 6–9. The present paper (a) examines the effects of factors 1, 4, and 6–9 on method development and (b) considers how to minimize their potentially adverse impact. While the present procedure assumes intervention and decision making by the chromatographer during method development, fully automated method development can be envisioned as part of an expert system [12,13].

2. Experimental

2.1. Equipment and materials

The HPLC system has been described elsewhere [14]. Solvents were of HPLC grade. The column was a 15×0.46 cm, 5-μm Zorbax StableBond C_8; the temperature was 30°C; the flow-rate was 2.0 ml/min.

2.2. Sample

A model 11-component sample was formulated from the following components (Aldrich,

Table 1
Factors to consider in the selection of an HPLC method development procedure and initial experimental conditions

Requirement	Comment
1. Method development should require a minimum number of runs	'Easy' separations developed with only a few runs; 'difficult' separations make use of the same initial runs, so no runs are wasted; each run evaluated before making a decision on further runs (sequential experiments)
2. Maximum range in α	More important for small N
3. α continuously variable	Minimizes number of runs required, especially with a computer
4. No problems in peak matching (tracking)	Especially important when a computer is used for retention mapping
5. α and R_s predictable from a minimum number of runs	Number of runs required varies with experimental condition that is varied
6. Experimental convenience and simplicity	Minimum number of mobile phase components; fast column equilibration between runs
7. Rugged final method	Separation insensitive to small changes in conditions (especially pH)
8. Applicable for most samples	Same method development approach, same experimental conditions (very important for samples of unknown composition)
9. Acceptable peak shape and plate number	Initial conditions selected to avoid wide or tailing peaks

Milwaukee, WI, USA): benzyl cyanide (1), *p*-cresol (2), 2-chloroaniline (3), 4-ethylaniline (4), N-ethylaniline (5), 3,4-dichloroaniline (6), 2-nitrotoluene (7), 3-nitrotoluene (8), toluene (9), 3-nitro-*o*-xylene (10), 4-nitro-*m*-xylene (11). This sample was selected to encompass a wide range of sample structures that can be separated isocratically.

2.3. Ternary-solvent mobile phases

Varying solvent type

Binary-solvent mobile phases of comparable strength were first formulated from organic plus buffer (2 m*M* potassium phosphate, pH 7.0). At this pH, the acidic and basic compounds in the sample are non-ionized. For each organic solvent B [acetonitrile (ACN), methanol (MeOH), tetrahydrofuran (THF)], a high and low %B mobile phase was prepared: 34 and 47% ACN, 47 and 61% MeOH, 30 and 39% THF. Ternary-solvent mobile phases were next formulated by mixing varying proportions of the high or low %B binaries; e.g., 34% ACN (B) plus 47% MeOH (B′) were blended as 12.5, 25, 37.5, 50, 62.5, 75 and 87.5% B–B′ mixtures.

Each of the six ternary-solvent blending studies were carried out at the same time, but the entire study was completed over a longer period. Replicate experiments for a given binary, e.g., 34% ACN, showed small shifts in retention (1–2%) between the two blending studies that shared that mobile phase, but little change in relative retention. Presumably these retention shifts reflect changes in the column over time or between columns.

2.4. Computer simulation

Isocratic separation as a function of any mixture of buffer and two of the three organic solvents was predicted from separations with the above mobile phases, using multi-parameter computer simulation [15] (DryLab for Windows software; LC Resources, Walnut Creek, CA, USA). These predictions were compared with experimental data for binary- and ternary-solvent mixtures to evaluate the accuracy of computer simulation as a function of the number of experiments used for simulation. Similar computer simulations were used to examine other questions related to HPLC method development and to illustrate the approach of Fig. 1.

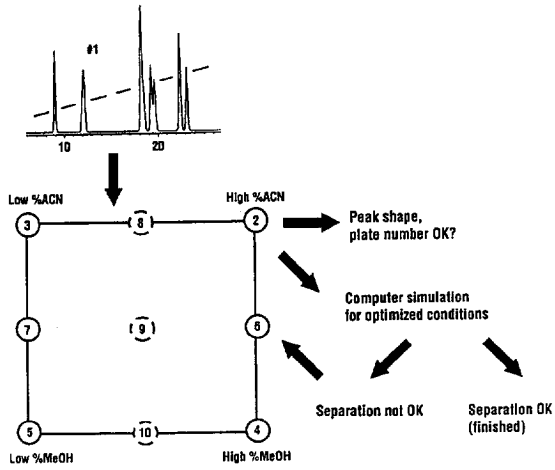

Fig. 1. Proposed approach to initial HPLC method development. Run 1 uses gradient elution; runs 2 to 10 are isocratic separations. See text for details.

3. Results and discussion

3.1. Proposed procedure for initial method development

A series of recommended experiments for reversed-phase method development is outlined in Fig. 1. An initial ACN–buffer gradient run (run 1) is used to characterize the sample in terms of reversed-phase (RP) retention; in some cases, isocratic RP separation will not be possible and method development must be pursued using another approach. Assuming that isocratic RP separation is possible, run 1 is used to estimate the composition of isocratic runs 2 and 3 (acetonitrile–buffer). On the basis of runs 2 and 3, computer simulation allows prediction of conditions for the best possible separation where %-ACN and column conditions (column dimensions, particle size, and flow-rate) are varied. If this procedure does not result in an acceptable separation, runs 4 and 5 (methanol–buffer) are

carried out next. Transfer rules can be used to estimate values of %-MeOH for these two runs, based on the results of runs 2 and 3 [4 (p. 32),16]. Computer simulation is continued with runs 4 and 5 to predict conditions for the best possible separation of the sample as a function of %-MeOH and column conditions.

If an adequate separation is not achieved on the basis of runs 2–5 with computer simulation, the critical band-pair (smallest R_s value) is compared between the ACN and MeOH runs. If the critical band-pair is not the same for these two solvents, then a separation with some mixture of MeOH and ACN may be possible. Runs 6 and 7 are carried out next, by blending equal volumes of the mobile phases for runs 2 plus 4 (run 6) and runs 3 plus 5 (run 7). Finally, these six runs (runs 2–7) allow predictions of retention and separation for mobile phases containing different proportions of ACN, MeOH, and water.

As an alternative to sequential experiments with intermediate interpretation, runs 2–7 of Fig. 1 can be carried out simultaneously. However, "easy" separations are able to be completed in as few as three runs (plus a final verification run), which means that carrying out runs 2–7 initially would result in more experiments than are necessary. A more important reason for sequential method development as advocated here, is that problems with either poor peak shape or a low plate number may be observed for one or more of runs 2–7. In this case, a change in conditions to restore good column performance [17] should be explored before further investigating separation as a function of ACN and MeOH concentrations.

If runs 1–7 of Fig. 1 do not result in a successful separation, then additional variables (pH, column type, ion-pairing, etc.) can be explored [2–6,10,11,15]. The results of runs 1–7 will prove useful as a starting point for these further studies. A similar procedure as in Fig. 1 can be used for samples which require gradient elution for the final method [18].

Why this choice of variables?

Most previous computer-assisted HPLC method development procedures have started with 7–15 (or more) experiments prior to data interpretation and optimization of the separation. This approach can work well for hard-to-separate samples that contain a large number (e.g., >10) of components. However, typical samples for HPLC separation are less demanding and require fewer experiments. These prior procedures [3–9,11–13] have also stressed the need for large, continuous changes in α as one or more experimental conditions are varied (again assuming hard-to-separate samples). Table 1 lists several other requirements (no. 1, 4–9) that deserve consideration in "practical" HPLC method development but which are often ignored. Table 2 evaluates the requirements of Table 1 for the more important variables used to control RP selectivity. To summarize Table 2, with the exception of solvent-strength and solvent-type selectivity based on MeOH and ACN, every other variable listed here can have significant practical disadvantages when used for method development. The use of solvent strength (%B) for controlling selectivity is somewhat limited, but in combination with solvent-type selectivity it is of proven value [8]. Table 2 therefore suggests that initial experiments in RPC method development are best restricted to a single column and mobile-phase mixtures of ACN, MeOH, and water. A similar (but non-sequential) approach has been described previously [19], but its justification was limited to a better control of run time (an additional advantage!) rather than attention to the various problems of Table 2.

3.2. Design and use of the initial gradient run

Choice of experimental conditions

The analysis of the preceding paper [1] plus other considerations allows a rational selection of preferred conditions for the initial gradient run (experiment 1 of Fig. 1). Table 3 lists some factors that are pertinent to this choice of initial conditions. It is desirable for subsequent isocratic runs that (a) no change in the column or flow-rate is required (no. 3 of Table 3) and (b) the column pressure does not exceed 2500 p.s.i. (=1724 MPa) (p. 12 of Ref. [6]). If MeOH–water mobile phases are to be used with 0.46 cm

Table 2
Potential limitations for different selectivity-controlling variables

Variable	Limitation[a]
%B	Limited selectivity range for many samples (no. 2)
Solvent type (ACN, MeOH, THF)	THF is unstable and requires a longer time for column equilibration (no. 6)
pH	Peak tracking is more difficult (no. 4); more experiments are required for computer simulation (no. 5); the final method may not be rugged (no. 7); only applicable to acidic or basic samples (no. 8); change in pH may adversely affect peak shape (no. 9)
Ion-pair reagent	Slow column equilibration, more complex, baseline problems common (no. 6); only applicable to acidic or basic samples (no. 8); peak shape problems common (no. 9)
Column type (C_8 or C_{18}, phenyl, cyano)	Selectivity is not continuously variable, which greatly reduces the value of changing columns (no. 3, no. 5); peak tracking can be more difficult (no. 4); less convenient than use of other variables (no. 6)
Temperature	Limited selectivity range for many samples (no. 2)

[a] no. 1, no. 2, ... refers to numbered items in Table 1, e.g., (no. 2) refers to 'maximum range in α' (no. 2 of Table 1); i.e., the maximum change in α with this variable is likely to be small.

Table 3
Goals that influence the choice of experimental conditions (t_G, V_m, F, $\Delta\varphi$, etc.) for the initial gradient run

Goal	Comment
1. Maximize resolution of first and later separations	Favored by long column and large t_G
2. Minimize run time, especially of later runs	Favored by short column and high flow-rate, small t_G
3. Minimize need for change of column or flowrate during method development	Anticipate change in pressure for water–methanol mobile phases
4. Accurate predictions	Favored by conditions such that $k = k^*$
5. Minimize effect of V_D	Favored by large t_G and F

I.D. columns containing 5-μm particles, then column length L (cm) and flow-rate F (ml/min) are constrained by the relationship (see Appendix A)

$$LF < 30 \quad (1)$$

When changing the mobile phase during HPLC method development as in Fig. 1, the column must be equilibrated before data are collected. This requires 10–15 column volumes of the new mobile phase followed by duplicate injections of the sample to confirm constant retention times. Assuming that k for the last band can be as large as 20, 50–60 column volumes will then be required for the total experiment. For a 25-cm column and 1.2 ml/min (Eq. 1), this corresponds to about 2 h per experiment and a plate number $N \approx 16\,000$. The use of a 15-cm column and 2 ml/min, on the other hand, requires no more than 45 min per experiment ($N \approx 8000$). Thus, the relative importance of time versus sample resolution (factors 1 versus 2 of Table 3) will suggest one or the other of these column length/flow-rate options. In our experience, shorter columns ($L < 15$ cm) often do not provide sufficient resolution for efficient method development (the recently described advantages of 3.5-μm particles [20] in lengths of 7.5 or 15 cm suggest these columns as possible alternatives for 15- and 25-cm, 5-μm columns, respectively).

Table 4 provides additional information relating to factors 4 and 5 of Table 3. The choice of column length, flow-rate, and gradient time for the first run affects the accuracy of isocratic predictions based on this run. Errors in predicted values $[(\%B)_{est}]$ of %-ACN for runs 2 and 3 (Fig. 1) are listed as a function of experimental conditions, band k-values and uncertainty in the gradient system dwell volume (V_D). If a value of V_D is not known, large errors in $(\%B)_{est}$ can result for flow-rates <2 ml/min.

On the basis of Table 4 and the above analysis of factors 1–3 of Table 3, we recommend a 15-cm column, 2 ml/min, and a 5–100% ACN–water gradient in a time $t_G = 60$ min. These conditions should result in predicted values of $(\%B)_{est}$ that

Table 4
Error in $(\%B)_{est}$ as a function of experimental conditions. Effect of uncertainty (if any) in dwell volume V_D

Conditions[a]			Error in $(\%B)_{est}$ for different k (1 S.D.)[b]			V_D error[c]	P_{max}[d]
L (cm)	F (ml/min)	t_G (min)	$k = 0.5$ (%)	$k = 5.0$ (%)	$k = 20$ (%)		
5	1	20	3.5	0.9	1.4	7.1	440
5	2	20	4.7	2.0	0.3	3.6	870
5	1	60	5.4	2.7	0.4	2.4	440
5	2	60	6.5	3.9	1.6	1.2	870
15	1	20	1.7	1.0	3.2	7.1	1300
15	2	20	2.8	0.2	2.1	3.6	2600
15	1	60	3.6	0.9	1.4	2.4	1300
15	2	60	4.7	2.0	0.2	1.2	2600
25	1	20	0.9	1.8	4.1	7.1	2170
25	2	20	2.0	0.6	2.9	3.6	4340
25	1	60	2.7	0.0	2.2	2.4	2170
25	2	60	3.8	1.3	1.1	1.2	4340

[a] Assumes 4.6 cm I.D. column; 5-μm particles.
[b] Values from Eq. 8 of Part I; $\delta S = 0.8$.
[c] Assumes the maximum error $\delta V_D \approx 3$ ml.
[d] Maximum column pressure (45% methanol–water and ambient temperature).

are sufficiently accurate for further method development.

Interpreting the initial run

Fig. 2 illustrates some possible outcomes of the initial gradient run. The preceding paper [1] provides a quantitative basis for distinguishing samples that can be carried out isocratically (Fig. 2a) from those that require gradient elution (Fig. 2b). Early-eluting samples (Fig. 2c) may require a change of pH, the addition of an ion-pair reagent, or the use of normal-phase HPLC. Late-eluting samples (Fig. 2e) may benefit from non-aqueous reversed-phase (NARP) or normal-phase HPLC. Samples with too many components for any single HPLC separation (Fig. 2d) require a preliminary separation or sample pretreatment prior to the use of a final HPLC procedure. It is important to recognize that samples such as those represented in Figs. 2c–2e require special handling, before continuing with the method development approach of Fig. 1.

3.3. Predicting retention for ternary-solvent mobile phases by computer simulation

The experimental design of Fig. 1 allows the prediction of separation as a function of mobile phase composition, based on experimental runs 2–7. Thus, starting with runs 2 and 3 (or 4 and 5), it is possible to predict retention k as a function of the volume fraction φ of organic in the mobile phase [21,22]:

$$\log k = \log k_w - S\varphi \quad (2)$$

Here k_w, S and φ are defined in part I [1]. Similarly, given runs 2, 4 and 6 (or 3, 5 and 7), it is possible to predict retention for intermediate ternary-solvent compositions. Several studies [9,11,19,22,23] suggest that ternary-solvent retention can be represented by

$$\log k = A + Bx + Cx^2 \quad (3)$$

Here, x is the volume fraction of one binary-solvent mixture in the final mobile phase; e.g., for $x = 0.5$, equal volumes of the mobile phases for runs 2 (high %-ACN) and 4 (high %-MeOH) are combined. The use of Eqs. 2 and 3 with runs 2–7 of Fig. 1 therefore allows prediction of separation for any intermediate mobile-phase composition.

Some workers have questioned the accuracy of Eqs. 2 and 3 for ternary solvent mixtures [23,24], hence bringing the approach of Fig. 1 into question. We therefore carried out the experiments described in the Experimental section as a means of further testing the reliability of Eqs. 2 and 3 for general application.

Varying solvent type

Fig. 1 assumes approximately equal-strength (isoeluotropic) ternary-solvent mobile phases for runs 2, 4 and 6 or 3, 5 and 7. We therefore evaluated Eq. 3 and a similar relationship,

$$k = A' + B'x + C'x^2 \quad (3a)$$

for their ability to predict retention, assuming experimental (input) runs with values of $x = 0$, 0.5 and 1.0. Ternary solvents based on ACN–MeOH, ACN–THF, and MeOH–THF were each evaluated at two different solvent-strength (%-water) levels.

The sample and mobile phases described in the Experimental section were used to obtain the retention data of Tables 5–7. The agreement of these data with Eq. 3 is summarized in Table 8. The mobile phases used to calculate the values of A, B, and C in Eq. 3 were in every case 0, 50% and 100% B, corresponding to the use (as in Fig. 1) of the two binary-solvent mobile phases and the 50:50 blend of these mixtures. For ACN–MeOH–water mobile phases, the average error in predicted retention times (for $x = 0.125$, 0.25, 0.375, 0.625, 0.75, and 0.875) was 0.6%. This corresponds to an average error in resolution R_s of 0.1 unit for $N = 8000$ (15-cm column, 2 ml/min), and a maximum error of 0.7 R_s units. We consider this acceptable accuracy for the prediction of separation based on Eq. 3 and the procedure of Fig. 1. The data of Tables 5–7 were correlated with Eq. 3a about as well as with Eq. 3.

The accuracy of predicted retention times was poorer for MeOH–THF–water and ACN–THF–water mobile phases, as seen in Table 8. The

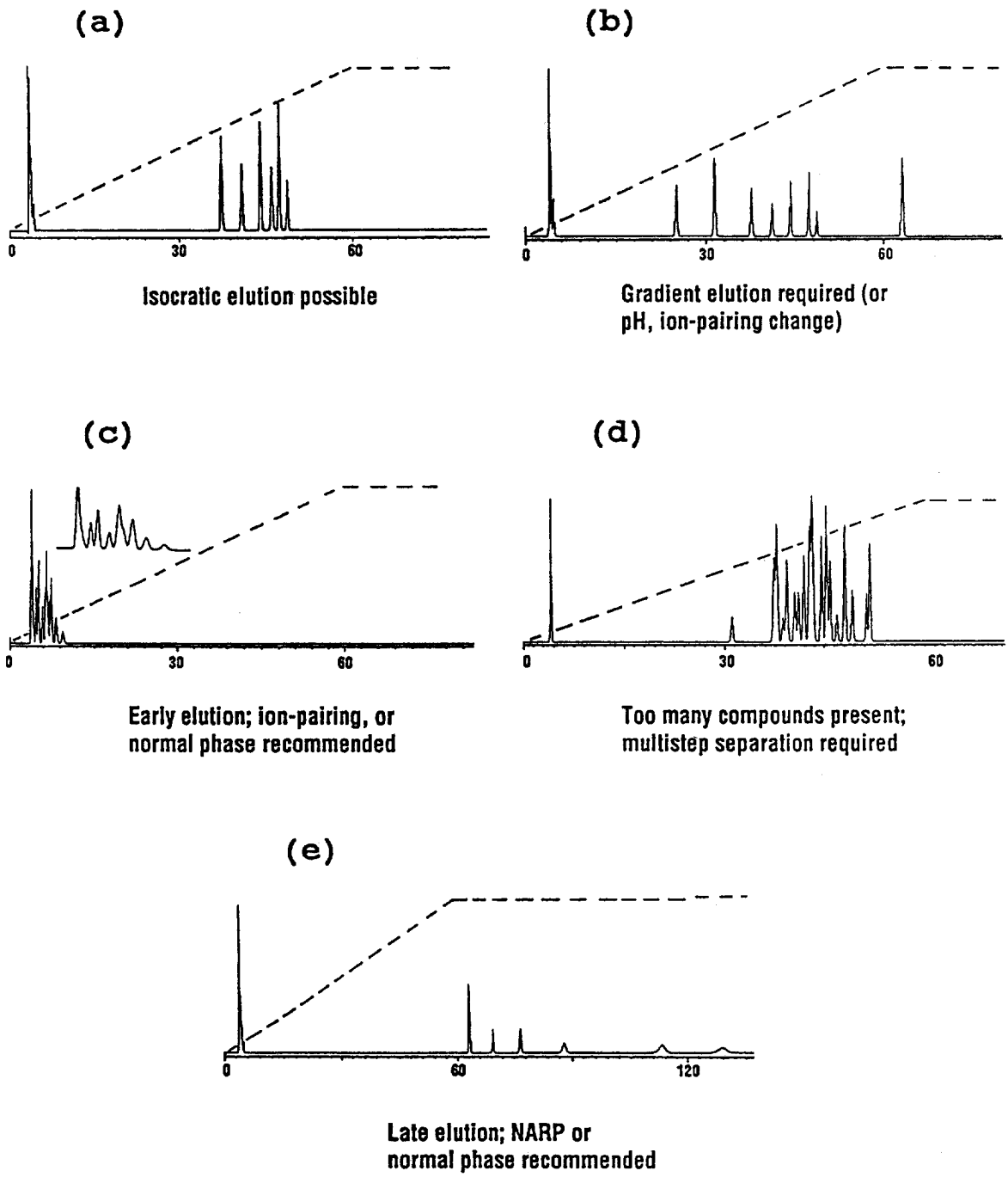

Fig. 2. Possible outcomes of an initial gradient run.

Table 5
Retention as a function of mobile-phase composition. Ternary-solvent mobile phases A–B are mixtures of binary-solvent mixtures ACN–water and MeOH–water of similar strength

Solute[a]	Retention times for indicated %B (min)								
	0	12.5	25.0	37.5	50.0	62.5	75.0	87.5	100.0
Mixtures of 34% ACN–water (A) with 47% MeOH–water (B)[b]									
1	12.07		11.51		10.59		9.24		7.95
2	8.56		8.98		9.16		8.74		8.00
3	12.21		11.74		10.98		9.78		8.57
4	12.33		12.37		12.09		11.08		9.78
5	17.73		16.94		15.84		13.93		11.82
6	21.01		21.57		20.57		17.78		14.75
7	22.78		22.49		21.17		18.54		15.82
8	24.98		24.92		23.68		20.85		17.79
9	27.10		26.20		24.79		22.61		20.64
10	35.93		36.77		35.21		30.73		25.72
11	37.96		39.37		38.41		34.19		29.06
Mixtures of 46% ACN–water (A) with 61% MeOH–water (B)[b]									
1	6.68	6.45	6.26	6.03	5.76	5.48	5.15	4.88	4.62
2	5.26	5.28	5.33	5.35	5.31	5.22	5.08	4.88	4.71
3	6.93	6.72	6.56	6.35	6.11	5.85	5.55	5.26	5.00
4	6.94	6.83	6.77	6.66	6.49	6.27	5.98	5.68	5.39
5	9.12	8.84	8.60	8.30	7.94	7.54	7.07	6.59	6.16
6	9.14	9.18	9.13	9.92	8.56	8.10	7.52	6.94	6.40
7	10.20	10.09	9.93	9.63	9.23	8.75	8.18	7.62	7.12
8	10.92	10.87	10.75	10.48	10.07	9.56	8.95	8.33	7.75
9	12.15	11.89	11.62	11.26	10.85	10.41	9.90	9.42	9.00
10	13.60	13.72	13.64	13.31	12.75	12.03	11.15	10.25	9.42
11	14.24	14.45	14.46	14.20	13.69	13.00	12.11	11.17	10.27

[a] See Experimental section for numbering of solutes.
[b] For example, if 250 ml of 34% ACN–water are mixed with 750 ml of 47% MeOH–water, the mobile phase is described as 25% B and the retention time for solute #1 is 11.51 min.

procedure of Fig. 1 would therefore give marginal or unacceptable accuracy in computer simulation, if THF were substituted for either ACN or MeOH. If five experimental runs (0, 25%, 50%, 75% and 100% B) are used instead of the present three runs, Eq. 3 can be applied to the 0–50% B and 50–100% B mobile phases separately with good results; i.e., acceptable precision of predicted retention times. However, this approach (using THF) requires additional runs. A similar observation has been reported earlier [25].

A simpler version of Eq. 3,

$$\log k = A + Bx \qquad (3b)$$

has also been reported [26]. This relationship is not sufficiently accurate for predicting an optimum value of x in ternary-solvent mixtures, but it can be useful for preliminary estimates of separation as a function of x. Thus estimates based on Eq. 3b, using runs 2 and 4 or 3 and 5 can be used to determine whether an investigation of ternary-solvent mobile phases is worthwhile.

Varying solvent strength

The accuracy of Eq. 2 is sufficient to predict separation as a function of %B for binary-solvent mobile phases when $15 < \%B < 85$. Its applicability for ternary-solvent mixtures has so far not been demonstrated and in fact has been questioned [19,22]. For this reason, we have

Table 6
Retention as a function of mobile-phase composition. Ternary-solvent mobile phases A–B are mixtures of binary-solvent mixtures THF–water and ACN–water of similar strength

Solute[a]	Retention times for indicated %B (min)								
	0	12.5	25.0	37.5	50.0	62.5	75.0	87.5	100
Mixtures of 30% THF–water (A) with 34% ACN–water (B)									
1	11.07	10.54	10.23	10.16	10.29	10.71	11.01	11.50	11.92
2	15.11	13.60	12.65	11.93	11.34	10.76	10.12	9.37	8.50
3	18.69	17.09	15.85	14.98	14.34	13.78	13.28	12.71	12.17
4	15.11	14.11	13.41	13.01	12.80	12.70	12.63	12.50	12.22
5	23.09	21.16	19.84	19.08	18.66	18.42	18.26	18.02	17.56
6	32.24	31.19	29.56	28.48	27.37	26.03	24.48	22.67	20.75
7	24.49	23.21	22.12	21.56	21.41	21.53	21.85	22.24	22.50
8	26.90	25.44	24.20	23.58	23.40	23.53	23.90	24.34	24.66
9	33.24	31.55	30.11	29.33	28.78	28.48	28.17	27.70	26.78
10	35.44	33.98	32.66	32.24	32.46	33.11	33.99	34.92	35.39
11	37.06	35.60	34.26	33.88	34.14	34.88	35.87	36.86	37.39
Mixtures of 39% THF–water (A) with 46% ACN–water (B)									
1	6.79	6.20	5.99	5.94	5.98	6.09	6.24	6.42	6.69
2	7.83	7.51	6.68	6.44	6.24	6.07	5.82	5.53	5.21
3	9.44	8.42	7.95	7.69	7.50	7.35	7.20	7.05	6.85
4	8.43	7.61	7.27	7.12	7.05	7.02	7.00	6.99	6.86
5	11.78	10.34	9.78	9.49	9.36	9.30	9.24	9.15	8.98
6	11.39	10.65	10.47	10.36	10.29	10.14	9.82	9.45	9.00
7	10.91	9.97	9.68	9.51	9.54	9.65	9.77	9.94	10.03
8	11.74	10.69	10.35	10.21	10.25	10.32	10.48	10.63	10.72
9	14.75	13.22	12.71	12.52	12.45	12.44	12.32	12.19	11.91
10	13.74	12.53	12.18	12.16	12.33	12.60	12.90	13.19	13.31
11	14.26	13.02	12.68	12.69	12.87	13.18	13.50	13.79	13.93

[a] See Experimental section for numbering of solutes.

tentatively broadened the method development scheme of Fig. 1 to include runs 8–10, which would allow values of k to be predicted from

$$\log k = A + B\varphi + C\varphi^2 \quad (4)$$

if necessary.

3.4. An example of method development according to Fig. 1

The data collected in the present study allow us to illustrate and evaluate the method development procedure of Fig. 1 for this particular sample. Computer simulations were used for this purpose, as summarized in Fig. 3. Run 1 was carried out using the gradient conditions recommended above. From the preceding paper [1], it can be determined that isocratic separation is possible; retention times for the first and last bands are 10 and 24 min, respectively, so that $1 < k < 10$. This same gradient run can also be used to select the %-ACN value for the next experiment. Because the accuracy of this prediction of %-ACN is best for $10 < k < 20$, we recommend that a %-ACN value be selected to provide $k = 10$ for the last band (run 2). According to Table 5 of the previous paper [1], a mobile phase of 40% ACN should give $k \approx 10$ for the last peak. Run 2 was carried out using 40% ACN and the other conditions of run 1 (last-band $k = 8$). Bands 1, 3 and 4 are observed to be poorly separated, so a further adjustment of

Table 7
Retention as a function of mobile-phase composition. Ternary-solvent mobile phases A–B are mixtures of binary-solvent mixtures THF–water and MeOH–water of similar strength

Solute[a]	Retention times for indicated %B (min)								
	0	12.5	25.0	37.5	50.0	62.5	75.0	87.5	100
Mixtures of 30% THF–water (A) with 47% MeOH–water (B)									
1	11.03	10.17	9.63	9.23	8.90	8.60	8.33	8.08	7.94
2	14.69	13.88	13.30	12.74	12.20	11.50	10.64	9.55	8.00
3	18.62	17.35	16.38	15.44	14.43	13.31	12.02	10.51	8.56
4	15.05	14.13	13.54	13.04	12.57	12.06	11.47	10.74	9.76
5	22.98	20.87	19.38	18.12	16.94	15.78	14.59	13.30	11.79
6	32.07	33.79	35.01	35.39	34.62	32.02	28.07	22.41	14.79
7	24.36	23.50	22.84	22.16	21.32	20.28	19.02	17.52	15.78
8	26.76	25.80	25.08	24.32	23.43	22.31	20.97	19.42	17.74
9	33.22	31.44	30.38	29.43	28.38	27.19	25.68	23.71	20.60
10	35.24	34.65	34.22	33.68	32.93	31.89	30.20	28.14	25.64
11	36.87	36.22	35.85	35.48	34.82	33.83	32.44	30.72	28.95
Mixtures of 39% THF–water (A) with 61% MeOH–water (B)									
1	7.01	6.34	5.95	5.65	5.41	5.18	4.97	4.77	4.59
2	8.18	7.62	7.28	6.95	6.61	6.20	5.78	5.29	4.69
3	9.90	9.11	8.63	8.13	7.61	7.02	6.38	5.72	4.97
4	8.78	8.10	7.72	7.38	7.05	6.68	6.30	5.86	5.36
5	12.44	11.10	10.33	9.61	8.94	8.25	7.57	6.86	6.12
6	12.20	12.43	12.72	12.71	12.27	11.33	10.01	8.48	6.36
7	11.51	10.92	10.57	10.19	9.74	9.17	8.54	7.83	7.06
8	12.38	11.79	11.44	11.03	10.55	9.93	9.25	8.48	7.68
9	15.43	14.53	13.98	13.44	12.79	12.04	11.21	10.22	8.92
10	14.65	14.06	13.77	13.41	12.91	12.22	11.39	10.42	9.32
11	15.20	14.61	14.35	14.01	13.54	12.88	12.09	11.16	10.16

[a] See Experimental section for numbering of solutes.

Table 8
Summary of errors for prediction of retention by Eq. 3a. Data of Tables 5–7, using 0, 50 and 100% B mobile phases to calculate values of A, B, and C

Ternary system	Average error[a] (%)		
	Retention time	Resolution R_s	
		$N = 8000$	$N = 16\,000$
ACN–MeOH	0.6 (2.5)	0.1 (0.7)	0.1 (0.5)
ACN–THF	2.0 (5.6)	0.3 (2.8)	0.2 (5.6)
MeOH–THF	2.6 (9.9)	0.7 (2.9)	0.5 (2.1)

[a] Maximum error in parentheses.

selectivity is required. Run 3 was carried out next, with 10% less ACN (30% ACN), to allow computer simulations as a function of %-ACN. This approach (10% decrease in %B for run 3 versus run 2 with $k = 10$ for the last band) will be adequate for most cases. If the retention range in run 1 is large, the retention of early bands may be too small ($k \ll 1$) in run 2, and a better approach may be the use of gradient elution. Eq. 11 from Part I [1] can be used to estimate the isocratic retention range as defined by the ratio $(k_z/k_a)_{max}$. Alternatively, the %B-values for runs 2 and 3 could be selected by using the estimated values of $\log k_w$ from run 1 (for $S = 4.2$) to predict separation as a function of %B (via computer simulation).

As seen in Fig. 3, bands 1, 3 and 4 are poorly

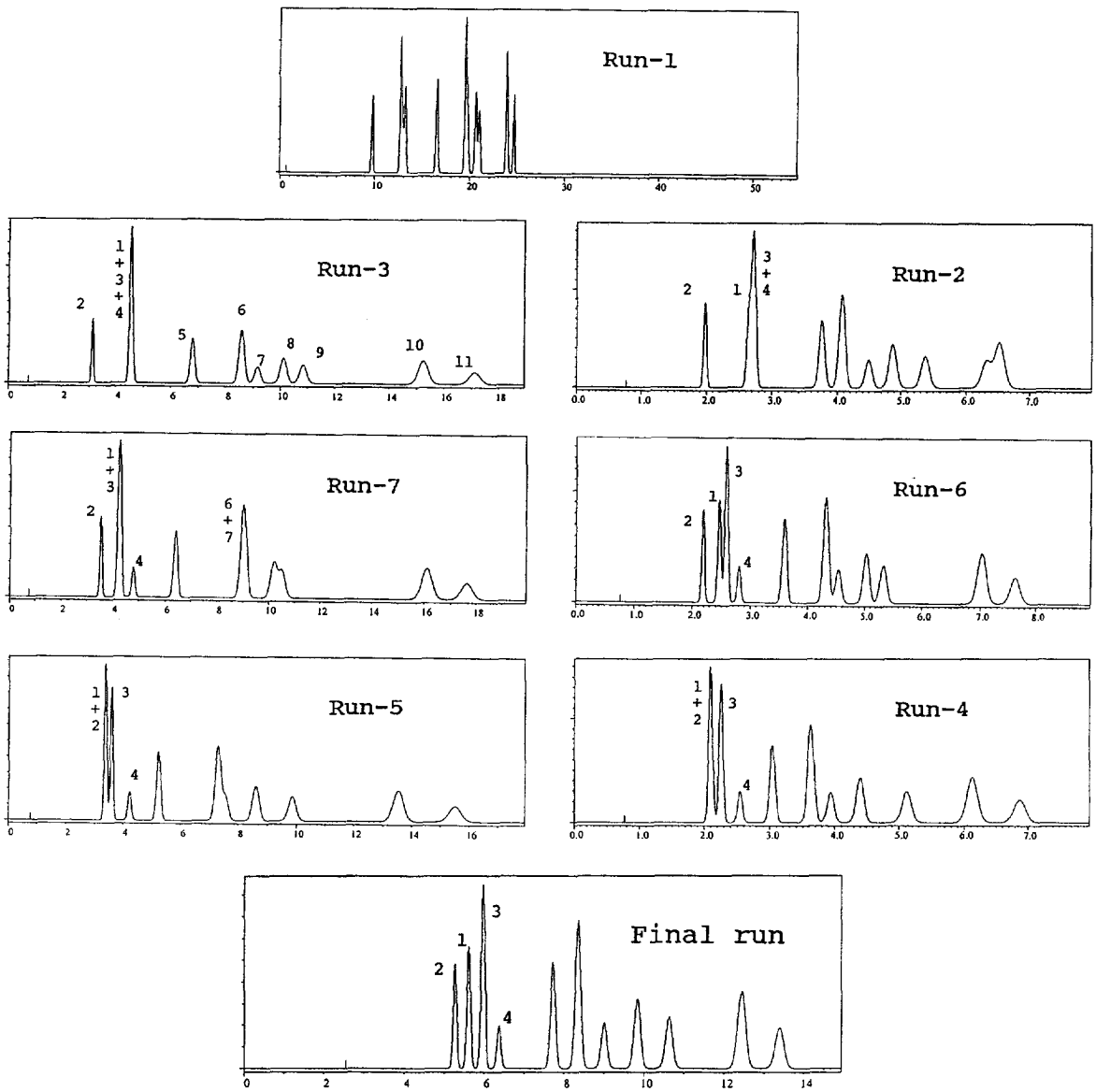

Fig. 3. Separation of present 11-component sample according to procedure of Fig. 1. Conditions: 15 × 0.46 cm, 5-μm C_8 column, 2.0 ml/min, 30°C. Run-1: 5–100% ACN–water gradient in 60 min; Run-2: 40% ACN; Run-3: 30% ACN; Run-4: 40% MeOH; Run-5: 50% MeOH; Run-6: 20% ACN + 25% MeOH; Run-7: 15% ACN + 20% MeOH. Final run: 23% ACN + 30% MeOH, 25-cm column at 1.0 ml/min ($R_s = 2.0$)

resolved for any value of %-ACN. A change in column conditions (column length, flow-rate, particle size) was also investigated (computer simulations) to no avail. Therefore, runs 4 and 5 using MeOH as solvent were carried out next, using Ref. [4] to predict appropriate values of %-MeOH (50% MeOH, run 4; 40% MeOH, run 5). Now bands 1 and 2 are unresolvable for any value of %-MeOH. Because the critical band-pair has changed between the ACN runs (bands 3/4) and the MeOH runs (bands 1/2), the use of ternary-solvent mobile phases appears promising.

The mobile phases from runs 2 and 4 are mixed 50:50 to give the mobile phase for run 6, and likewise for runs 3 and 5 to give run 7. The chromatogram of run 6 shows all 11 components partially resolved for the first time. Computer simulation can now predict separation as a function of any ACN–MeOH–water mobile phase; maximum resolution ($R_s = 1.3$) occurs for 23% ACN + 30% MeOH. Because this is short of baseline resolution ($R_s > 1.5$), column conditions were varied to give $R_s = 2.0$ (25-cm column and 1.0 ml/min flow-rate). This final run is shown in Fig. 3.

Interestingly, mixtures of THF with either MeOH or ACN were unable to achieve a separation as good as that found in Fig. 3. Fig. 4 shows resolution as a function of mobile-phase composition (ACN and MeOH mixtures, 25-cm column, 1.0 ml/min). The large circles correspond to runs 2–7 of Fig. 1, and the small circle is for the final run of Fig. 3. In this particular case, maximum resolution occurs for a relatively short run time (≈12 min). Fig. 4 is of further interest in connection with a proposed simplification of the procedure of Fig. 1. It has been suggested by Gazdag et al. [27] that an optimum ternary-solvent composition can be obtained by mixing the optimum binary-solvent mixtures. This approach is tested in Fig. 4 by connecting the optimum binary-solvent compositions (34% ACN, 62% MeOH) by the dashed diagonal line, corresponding to mixtures of these two mobile phases. The best resulting resolution is $R_s = 1.9$ in this case, which is sufficiently close to the true optimum ($R_s = 2.0$). This suggests that the Gazdag et al. strategy may be useful for other samples.

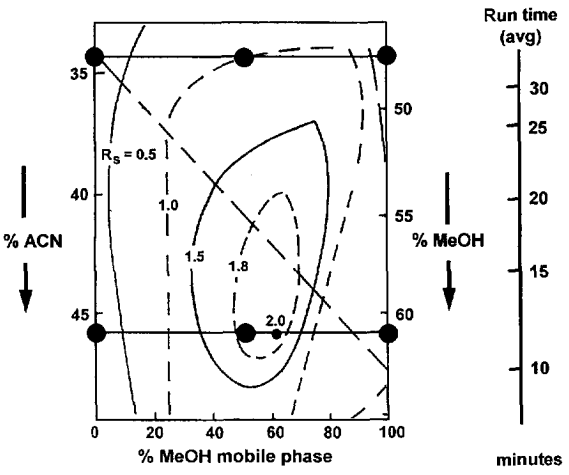

Fig. 4. Resolution map for present 11-component sample as a function of mobile-phase composition (ACN–MeOH–water mixtures). Conditions as in the Experimental section (25-cm column, 1.0 ml/min).

4. Conclusions

On the basis of the preceding paper [1], it is possible to select optimum experimental conditions for an initial method development experiment using gradient elution. These conditions (15 × 0.46 cm, 5-μm column, 2 ml/min, 5–100% acetonitrile–buffer gradient in 60 min) can provide an accurate prediction of the best %-acetonitrile to be used in the following isocratic run (run 2 of Fig. 1). These same conditions are also well suited for the subsequent runs 3–10 of Fig. 1.

Experiments to control selectivity and maximize sample resolution are best selected on the basis of several practical factors (Tables 1 and 2). The discussion of the present paper suggests that initial experiments should vary the composition of mobile phases composed of acetonitrile–methanol–buffer. It is believed that this approach (Fig. 1) will be suitable for the majority of samples requiring HPLC separation. When this proves not to be the case for a given sample, then additional variables can be explored (THF as solvent, pH, temperature, etc.) as described in Refs. [2–5].

The experimental plan of Fig. 1 can be carried out either all at once or sequentially with intervention by the chromatographer. The advantage of a sequential procedure is that it minimizes wasted runs. Thus, if inspection of run 2 of Fig. 1 shows broad or tailing bands, this problem should be solved before going on to runs 3–10. The present disadvantage of sequential runs is that the user is required to be present, which minimizes the advantage of unattended overnight operation. However, the development of

expert systems that are integrated into the HPLC system controller should eventually allow unattended, sequential method development as described here. We are presently working on such an expert system.

Appendix A. The role of pressure drop in selecting column conditions

The column pressure drop P is given as [28]

$$P = 140L^2\eta/t_o d_p^2 \quad \text{(A.1)}$$

where P is in psi, column length L is in cm, mobile-phase viscosity η is in cPoise, dead time t_o is in min, and particle size d_p is in μm. The column dead time (min) can be approximated as

$$t_o = V_m/F \approx 0.5Ld^2/F \quad \text{(A.2)}$$

where V_m is the column dead volume, d is the column internal diameter, and F is in ml/min. Combining Eqs. A.1 and A.2:

$$P = 280LF\eta/(d^2 d_p^2) \quad \text{(A.3)}$$

If the pressure is to be less than some maximum value P_{max}, then

$$LF < 0.0036 d^2 d_p^2 P_{max}/\eta \quad \text{(A.4)}$$

For a maximum viscosity of $\eta = 1.64$ (45% methanol at ambient temperature [29]), a column diameter $d = 0.46$ cm, 5-μm particles, and a maximum pressure $P_{max} = 2500$ psi (1724 MPa),

$$LF < 30 \quad \text{(A.5)}$$

List of symbols

A	water,
ACN	acetonitrile,
b	gradient steepness parameter (Part I, Ref. [1], Eq. 2),
b_1, b_2	different values of b; Part I, Ref. [1], Eq. C.4,
B	organic solvent in mobile phase,
c	Part I, Ref. [1], Eq. 6; $c = b/t_o$,
F	flow-rate (ml/min),
k	solute retention factor equal to $(t_R - t_o)/t_o$,
k_a, k_z	k-value of first (a) and last (z) bands in an isocratic separation; see Part I, Ref. [1], Fig. 2b,
k_o	value of k at the beginning of gradient elution (for $\varphi = \varphi_o$),
k_w	value of k for water as mobile phase ($\varphi = 0$),
$(k_z/k_a)_{max}$	maximum allowable value of the ratio k_z/k_a,
k^*	effective value of k in gradient elution (Part I, Ref. [1], Eq. 9),
L	column length (cm),
MeOH	methanol,
p, q	constants in Part I, Ref. [1], Eq. 4,
P	pressure drop across column (psi),
P_{max}	maximum allowable value of P,
S	constant in Part I, Ref. [1], Eq. 2; equal to $d(\log k)/d\varphi$,
t_D	gradient equipment dwell time (min),
t_G	gradient time (min),
THF	tetrahydrofuran,
t_o	column dead time (min),
t_R	solute retention time (min),
t_{Ra}, t_{Rz}	values of t_R for first (a) and last (z) bands in a gradient run; see Part I, Ref. [1], Fig. 2a,
T_R	Part I, Ref. [1], Eq. 6; $T_R = t_R - t_o - t_D$,
V_D	gradient equipment dwell volume (ml),
V_m	column dead volume (ml); equal to t_o/F,
x	parameter in Part I, Ref. [1], Eq. 5; $x = t_D/(t_o k)$,
δS	error in assumed value of S, equal to $S - 4.2$,
Δt_R	$t_{Rz} - t_{Ra}$; see Part I, Ref. [1], Fig. 2a,
δV_D	error in assumed value of V_D (if V_D is not known),
$\Delta\varphi_i$	difference in φ_i values for a solute as a result of a change in gradient steepness b (Part I, Ref. [1], Eq. C.4),
$\Delta\varphi$	change in φ during the gradient δ_{t_R},

φ volume fraction of organic in mobile phase; equal to 0.01 %B,
φ_i value of φ for a band at elution (gradient run),
φ_o value of φ at start of gradient,
η mobile phase viscosity (cPoise),
$(\%B)_{est}$ estimated value of %B to obtain a given value of k for some solute (based on initial gradient run).

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