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Systematic Approaches to HPLC Method Development for Reversed-Phase Separation†

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Several approaches to HPLC method development have been reported in the literature, many of which rely on computer software to minimize the total number of experimental runs. The major decision in method development is how best to vary band spacing for improved separation of a sample. The present report summarizes the present status of computer-assisted reversed-phase HPLC method development, and illustrates two possible approaches in more detail: (a) optimization of mobile phase composition for mixtures of acetonitrile, methanol and buffer; (b) optimization of separation temperature and either gradient steepness or isocratic %B.

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† Dedicated to Professor Edward Soczewiński on the occasion of his 70th birthday.

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** One of the authors (LRS) would like to take this opportunity to extend his deepest regard and affection for Professor Edward Soczewinski. On the occasion of his 70th birthday, it is appropriate for all of us to recognize and honor a lifetime of dedicated and productive work in the field of chromatography, but for me the matter is also more personal. Edward and I first exchanged correspondence in 1967, at the time he introduced his famous "Soczewinski equation" to the world. When I first read a preliminary draft of this paper, my initial reaction was one of skepticism. But some further testing of this concept quickly convinced me of both its validity and widespread utility. Edward was also so kind as to incorporate some comments of mine into this paper (E. Soczewiński, *Anal. Chem.*, **41**, 180 (1969)). Since that time, Edward and many other researchers have made good use of this relationship as a means of obtaining better separations and better understanding what is actually occurring in the column or on the TLC plate. Since 1968 Edward and I have become very good friends, with frequent meetings at international symposia and a long-standing and frequent correspondence on topics of mutual interest. Our shared scientific interests have been further cemented by my wifes search for her own Polish roots, which has occasioned visits to Poland and further opportunities to meet with Edward. This close interaction over the years has made me especially aware of his many good personal characteristics: kindness, loyalty, honesty, integrity, and a persistent good nature to name but a few. So, again I wish Edward all the best in his coming retirement and hope for many more happy years.

W literaturze opisano wiele sposobów opracowywania metodyk HPLC, w których często korzysta się z programów komputerowych w celu zminimalizowania całkowitej liczby eksperymentów. Głównym problemem przy opracowywaniu metodyki jest zmiana odległości pasm w celu polepszenia rozdzielenia składników próbki. Niniejsza praca podsumowuje obecny stan opracowywania metodyk HPLC w odwróconym układzie faz przy użyciu komputera ze szczególnym uwzględnieniem dwóch sposobów: (a) optymalizacja składu fazy ruchomej dla mieszanin acetonitrylu, metanolu i buforu; (b) optymalizacja temperatury rozdzielania przy stromym gradiencie albo przy izokratycznym %B.

The first objective in HPLC method development is to achieve adequate separation as measured by sample resolution R_s. Resolution for two adjacent bands in isocratic elution can be related to experimental conditions by (1)

$$R_s = (1/4)\ \ [k/(k+1)]\ \ (\alpha-1)\ \ N^{1/2} \tag{1}$$

where k is the solute retention factor, α is the separation factor, and N is the column plate number. A systematic approach to method development should first adjust conditions so that $0.5 < k < 20$ for every band in the chromatogram. The next step is to "optimize" band spacing or values of α for each band-pair. Finally, if resolution requires further improvement, or the run time needs to be shortened, N can be varied by changing either column length or flow rate.

For gradient separations, Eq. (1) can be replaced by

$$R_s = (1/4)\ \ [k^*/(k^*+1)]\ \ (\alpha\text{-}1)\ \ N^{1/2} \tag{1a}$$

where k^* is an effective retention factor in gradient elution. The value of k^* for each band in a gradient elution chromatogram will usually be similar (unlike values of k for isocratic elution), and k^* can be related to experimental conditions as

$$k^* = 0.87\ t_0\ \Delta\phi\ S/t_G \tag{2}$$

Here, t_0 is the column dead-time, $\Delta\phi$ is the change in the volume-fraction ϕ of the B–solvent during the gradient (ϕ = 0.01 %B), S is a characteristic solute parameter which for compounds with molecular weights < 500 has a value of about 4, and t_G is gradient time. In gradient elution, it is desirable that $0.5 < k^* < 20$, similar to the case for isocratic separation.

The above approach to method development is illustrated in Figure 1 by the isocratic separation of a mixture of 10 substituted benzenes. These and later examples are computer simulations based on actual experimental data and can be considered equivalent to "real" chromatograms ([1] chapter 10). In a first experiment (Fig. 1a), with a 15 × 0.46 cm C_8 column at 2 ml min^{-1}, a strong mobile phase (80% acetonitrile (ACN)/water) is used to ensure rapid elution of the sample. As expected, the sample leaves the column rapidly under these conditions, with little separation of adjacent peaks. The next step is to adjust solvent strength (%B = % ACN) so that $0.5 < k < 20$ for all peaks. A mobile phase of 40% ACN accomplishes this objective (Fig. 1b). However, band spacing is poor in that compounds 1–3 and 9–10 are not resolved to baseline (baseline separation requires $R_s > 1.5$). At this point, it is necessary to change band spacing. For some samples, this can be achieved most conveniently by adjusting

%B over a range (30–60% ACN for the sample of Fig. 1) that still provides $0.5 < k < 20$. However, in the present case no improvement in the separation of bands 1–3 results for any value of % ACN.

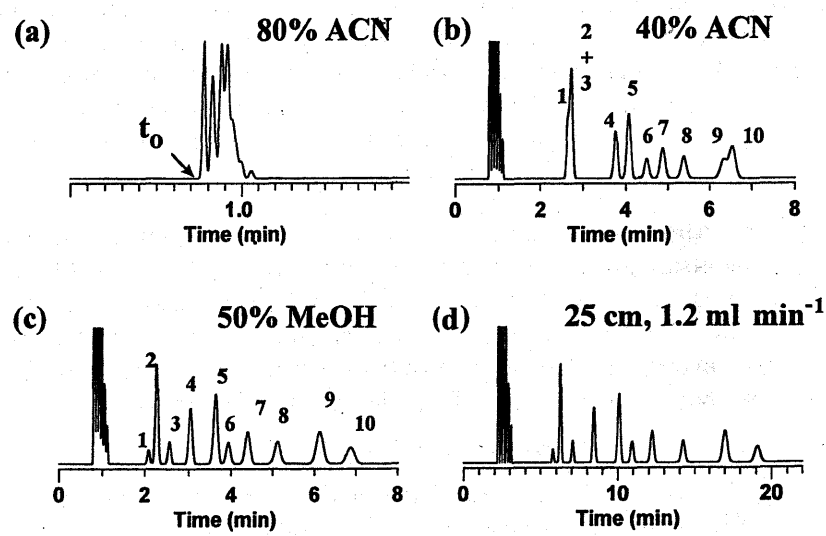


Figure 1. Method development for a 10–component sample. Computer simulations based on data reported in [4]. Sample: 1) benzonitrile; 2) 2-chloroaniline; 3) 2-ethylaniline; 4) *N*-ethylaniline; 5) 3,4-dichloroaniline; 6) 2-nitrotoluene; 7) 3-nitrotoluene; 8) toluene; 9) 4-nitro-*m*-xylene; 10) 4-nitro-*m*-toluene. Conditions: 15 × 0.46 cm C_8 column; ACN or MeOH/water mobile phases as indicated; 2.0 ml min^{-1}; 35°C

A more effective means of changing selectivity (values of α) is to change the B–solvent. In the present example, methanol (MeOH) was substituted for acetonitrile. The % MeOH must be adjusted upward to 50% MeOH (Fig. 1c) in order to achieve the same run time as for 40% ACN in Figure 1b. Band spacing and separation are much improved in the separation of Figure 1c, and the resolution of the "critical" (most overlapped) band-pair 1–2 is now $R_s = 1.4$. This is almost baseline resolution, but the continued use of the method is expected to result in a gradual loss in column plate number N and a decrease in resolution. For this reason, an initial resolution of $R_s \geq 2$ is desirable. At this point, a moderate further increase in resolution is needed, and this can be provided by an increase in column length. Figure 1d shows the final separation, where a 25 cm column has replaced the original 15 cm column, and the flow rate has been decreased to 1.2 ml min^{-1} to maintain column pressure < 2000 psi. The resolution of the critical band-pair 1–2 is now $R_s = 2.1$, which is acceptable for a routine procedure which can be applied to many samples.

The following discussion provides a more detailed, current examination of the above three steps in HPLC method development: adjusting conditions for (a) adequate sample retention ($0.5 < k < 20$), (b) adequate band spacing (α), and (c) increased resolution or shorter run time as a result of a change in column dimensions or flow rate. For further background on the present topic, see [1].

ADJUSTING SAMPLE RETENTION (k)

Following an initial isocratic separation with a strong mobile phase as in Figure 1a, it is necessary to adjust %B so as to achieve $0.5 < k < 20$ for all sample-compounds of interest. There are two procedures that can each shorten a trial-and-error search for a "best" value of %B. In one approach, the k-value for the last peak of the first experiment is determined; $k \approx 0.3$ in the example of Figure 1a. The average increase in k for a 10% decrease in B can be obtained from a general relationship [2]:

$$\log k = \log k_w - S\phi \tag{3}$$

Here, k_w is the (extrapolated) value of k for $\phi = 0$ (0% B). An average value of $S \approx 4$ can be assumed for sample molecular weights < 500 Da (3), so that a 10% decrease in B (0.1 decrease in ϕ) should result in an increase in log k by 0.4 units (*i.e.*, k increases by 2.5 fold). Our second experiment should provide for all peaks, which suggests a target k value for the last peak of $k \approx 10$ (or $\log k = 1$). Based on Eq. 3 with $S \approx 4$, we can estimate the value of %B for the second experiment as

$$\%B = (\%B)_1 + 25(\log k_1 - 1) \tag{4}$$

Here, $(\%B)_1$ refers to the value of %B in the first experiment, and k_1 is the value of k for the last band in the first experiment. For the example of Figure 1a, $k_1 = 0.3$ and $(\%B)_1 = 80$, so we can predict that %B for the next experiment should be: %B = 80 + 25(–0.53–1) = 43%. The actual value of k for the last band with a mobile phase of 43% B was $k \approx 6$, which is close enough for our purposes. It is useful to carry out a third experiment with either higher or lower %B, while maintaining $0.5 < k < 20$ for all bands, and to observe any change in selectivity with %B (see following section).

A second procedure for adjusting sample retention and %B is to start method development with an initial gradient run. This approach has several advantages:

- the initial separation suggests whether reversed-phase separation under either isocratic or gradient conditions will be feasible; *i.e.*, the sample is either unretained or retained too strongly
- the initial separation can be used to determine whether isocratic or gradient elution should be used for the final HPLC method (example given in a later section)
- the initial separation can provide an estimate of the best %B value for the second experiment, when isocratic elution is preferred
- the initial separation can provide estimates of the best initial and final %B values when gradient elution is preferred
- an initial gradient experiment makes it less likely that minor peaks that elute early or late will be overlooked

For a detailed further discussion of the use of an initial gradient run in method development, see ([1] chapters 8 and 9), [3,4].

OPTIMIZING SAMPLE SELECTIVITY (α)

In this second, most important phase of method development, the goal is to maximize the resolution of the least-resolved ("critical") band pair by maximizing its value of α. In order to minimize the number of experiments required to achieve an acceptable band spacing and values of α, attention must be given to two separate questions: (1) which experimental conditions should be varied to create changes in α, and (2) what specific experiments must be carried out so that optimized band spacing and resolution can be obtained?

Table 1 summarizes the most often used experimental conditions for creating changes in band spacing. It is possible to rank these variables in terms of their ability to provide maximum changes in selectivity [5], which makes it more likely that an initially overlapped band-pair can be separated by change of the variable:

any sample – temperature (least effective) < column type ≈ solvent strength solvent < type (most effective)

ionic sample – buffer or additive concentration (least effective) < pH ≈ ion-pair-reagent concentration (most effective)

Table 1. Some experimental conditions used to control selectivity

All samples	Ionizable samples (acids or bases)
solvent strength (%B)	pH
solvent type*	ion-pair-reagent concentration
column type**	buffer or additive concentration
temperature	

* Acetonitrile, methanol or tetrahydrofuran.
** Alkyl silica (C_8 or C_{18}), phenyl or cyano.

For "any sample", which can consist of ionic and/or neutral compounds, the preferred variable for controlling band spacing and maximizing resolution is solvent type. On this basis, it has been suggested [6,7] to vary the concentrations of acetonitrile, methanol and tetrahydrofuran (THF) in the mobile phase, while maintaining run time and values of k approximately constant by adjusting water content. By carrying out seven pre-selected experiments with varying mobile phase compositions, it is possible (using a computer) to predict a mobile phase that gives the largest "critical" resolution. This approach, when first proposed in 1980, excited immediate interest and was soon applied in many laboratories around the world. Later studies [2,8] showed, however, that accurate predictions of retention require more than seven (*e.g.*, 10–14) initial experiments.

For an "ionic sample", the preferred variables for controlling band spacing are pH and the concentration of an ion-pair reagent. On this basis, method development procedures have been reported for the optimization of separation through simultaneous changes in pH and ion-pair-reagent concentration [9,10]. Again, the use of a computer limits the number of experiments required to find the best values of pH and

ion-pair-reagent concentration. However, a full optimization of pH and ion-pair-reagent concentration will require >> 10 experiments for accurate computer predictions.

Although solvent type, pH and ion-pair-reagent concentration usually lead to maximum control over band spacing and the highest sample resolution, other factors are also important when choosing variables to control band spacing:

- completion of method development with the fewest experimental runs (presumably with the help of appropriate method development software)
- conditions (variables) that are convenient to change
- a final HPLC method that is rugged and convenient to carry out, preferably with a short run time
- a method development procedure which is applicable to as wide a range of sample types as possible; *e.g.*, both neutrals and ionics, samples that require low–UV detection, *etc.*

As discussed elsewhere [5,11,12], many variables are incompatible with all of the above goals; these latter, less favorable variables include pH, ion-pairing, column type and the use of THF as solvent. This is not to say that the latter variables cannot be useful for changing band spacing, but rather that these variables should not be our first choice in method development. What then is left? Two possibilities [1,11] are illustrated in Figure 2 for initial method development experiments: (Fig. 2a) variation of the composition of mobile phases that contain ACN, MeOH and/or water, or variation of temperature and either %B (Fig. 2b, isocratic) or gradient time (Fig. 2c). The latter two procedures are essentially similar in terms of their ability to optimize band spacing, because of the equivalence of gradient time and isocratic %B in controlling selectivity.

Each of the method development procedures of Figure 2 can usually achieve the separation of samples with fewer than 20 components [13]. Less demanding samples can be separated by changing only a single variable; *e.g.*, %B, pH, *etc.* Computer simulation (*e.g.*, using DryLab software) is well suited for optimizing a single variable [1], and has recently been extended to the method development procedures of Figure 2a-c.

Variation of ACN, MeOH and water (Isocratic Elution)

Figure 2a outlines six to nine experiments for varying the concentrations of ACN, MeOH and water in the mobile phase as a means of optimizing band spacing. Experiments 1–6 of Figure 2a are usually adequate for mapping separation as a function of mobile phase composition. In order to ensure maximum accuracy in computer predictions, additional experiments 7–9 may be needed for mobile phases that cover a wider range in % organic, or which contain lower (0–20%) or higher (80–100%) water. The mobile phase compositions shown in Figure 2a are representative and must be varied for different samples. To determine these initial mobile phase compositions for each sample, one or more experiments are required to determine the solvent strength required for $0.5 < k < 20$.

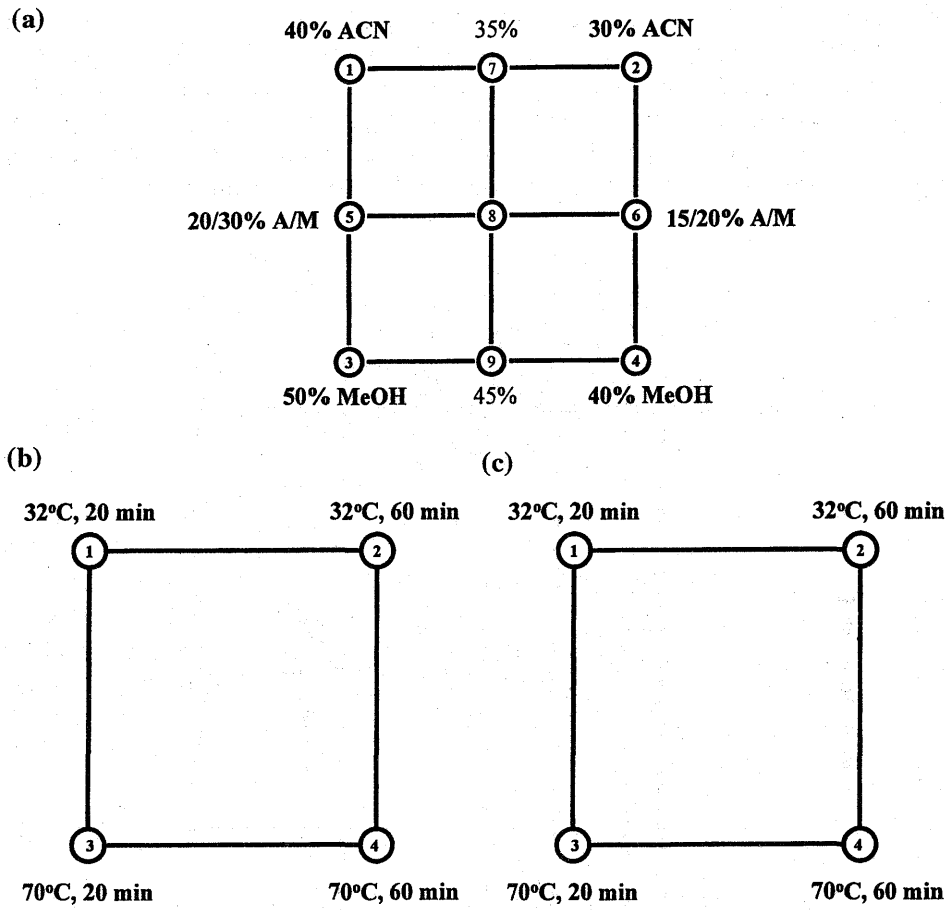


Figure 2. Two preferred approaches to optimizing band spacing for reversed-phase HPLC. (a) variation of solvent type and strength (isocratic mobile phase mixtures of ACN, MeOH and water); (b) variation of temperature and isocratic solvent strength (%B); (c) variation of temperature and gradient time. Reprinted from [1] with permission

It will be assumed that a computer is used with the six-nine experiments of Figure 2a, in order to predict separation as a function of any mobile phase composition and arrive at conditions for maximum R_s. The application of this method development procedure to an 11-component sample is illustrated in Figure 3. A first separation (a) is carried out for a 5–100% ACN/water gradient, in order to predict a solvent strength (40% ACN) [3] that will provide $0.5 < k < 20$ under isocratic conditions. The experimental isocratic separation (run 2 with 40% ACN) is shown in (b). Bands 1, 3 and 4 are overlapped completely, and bands 10 and 11 are partially overlapped. So it is necessary to improve band spacing for increased sample resolution.

In pursuit of the 6 run procedure outlined in Figure 2a, successive experiments (1–6) are carried out with a lower % ACN – 30% ACN in (c), higher and lower % MeOH – 50% MeOH in (d), 40% MeOH in (e), and mixtures of ACN and MeOH:

mobile phases (b)+(d) (Fig. 3f) and (c)+(e) (Fig. 3g). Bands 1 and 2 are unseparated in the MeOH runs (d,e), but are separated in the ACN runs (b,c). This suggests a mixture of ACN plus MeOH for the mobile phase, as confirmed in Figs. 3f and g. All 11 bands are separated in the chromatogram of Figure 3f (stronger mobile phase, shorter run time). Since separation is poorer in Figure 3g (weaker mobile phase, longer run time), the use of a stronger mixture of these two solvents (lower % water)

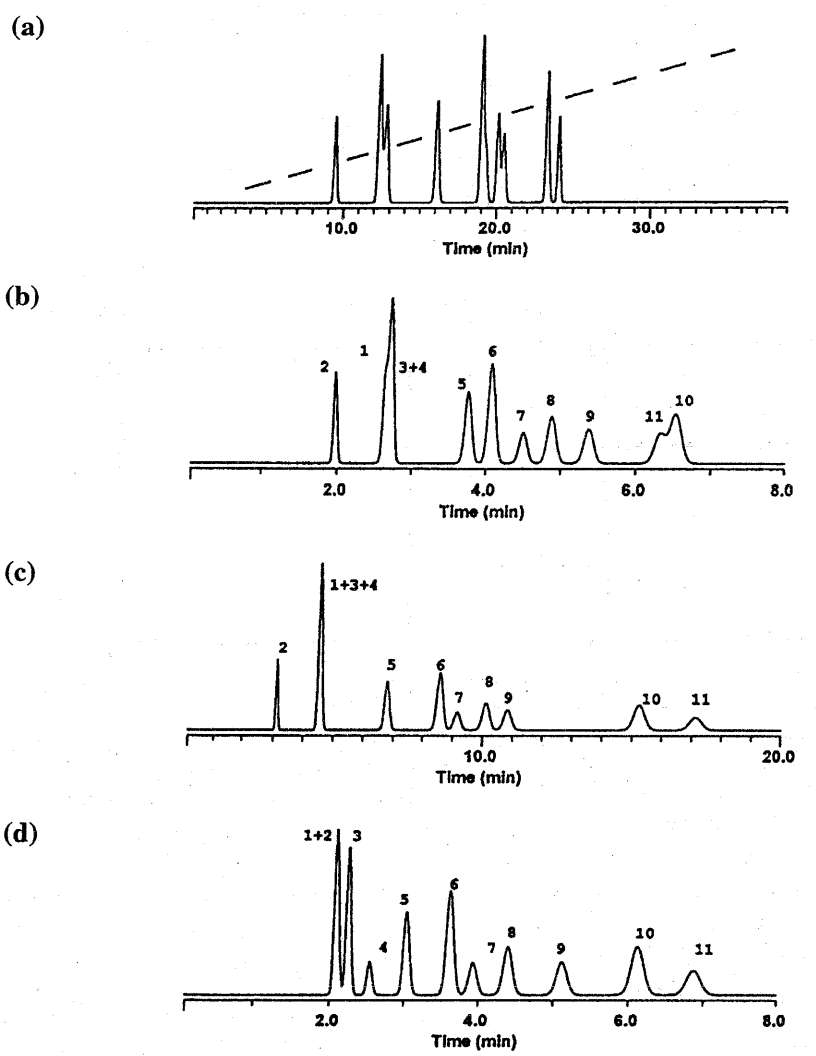


Figure 3. Method development for an 11-component sample according to plan of Figure 2a (beginning with a gradient separation). Computer simulations based on data reported in [4]. Sample: same as in Figure 1 plus *p*-cresol (compound 2). Conditions as in Figure 1 except for use of different mobile phases: (a) 5–100% ACN in 60 min; (b) 40% ACN; (c) 30% ACN; (d) 50% MeOH; (e) 40% MeOH; (f) 20% ACN + 25% MeOH; (g) 15% ACN+ 20% MeOH; (h) same as (g), except 25 cm column and 1.0 ml min^{-1} (see figures e –h on next page)

is indicated. For these latter conditions, computer simulation predicts the optimum separation of Figure 3h, where all bands are separated with R_s=1.3.

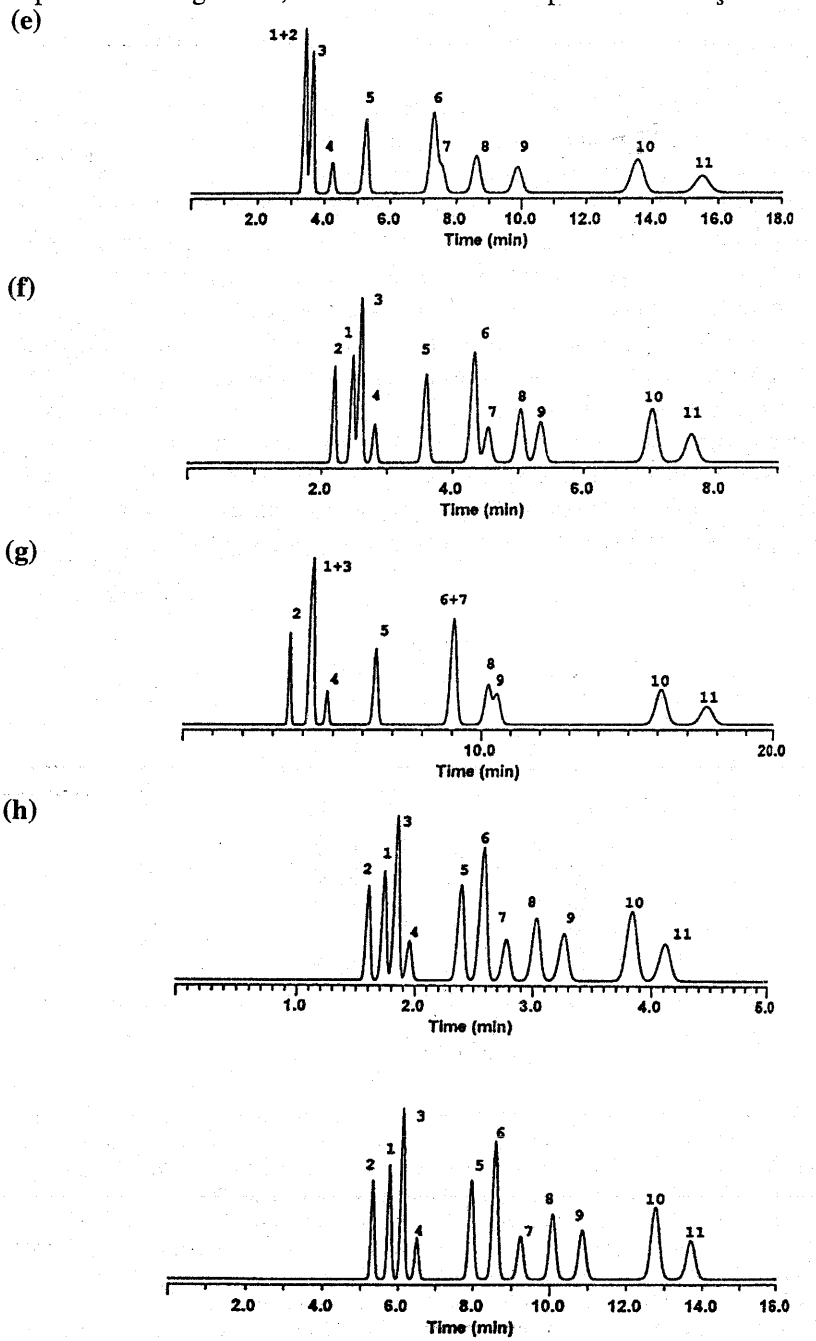


Figure 3. (continuation).

Variation of temperature and gradient time

The four runs required for this approach are illustrated in Figure 2c. The conditions of temperature and gradient time shown in the figure are representative and can be varied, but these particular conditions are suitable for any sample. Commercial software (DryLab *for Windows*, Version 2.0; LC Resources) allows the prediction of separation as a function of temperature, gradient conditions (gradient time, initial and final %B in the gradient, gradient shape) using the four runs of Figure 2c. We will illustrate this approach to method development, using a 17-component sample that contains various substituted anilines and benzoic acids. Figures. 4a–d show the four experimental runs used for computer simulation. In the chromatograms of Figures 4a,b, there are two critical band-pairs that are totally overlapped: 8,9 and 11,12. In Figure 4c, the critical pair is 10,11 (partially overlapped), and in Figure 4d the critical pair is 4,7 (totally overlapped). The best separation is that of Figure 4c, with R_s= 0.6.

Using the 2-dimensional resolution map provided by the DryLab software, it is possible to select a temperature and gradient time that provides maximum resolution (assuming no change in the initial gradient range of 5–65% ACN). This resolution map for the present sample is shown in Figure 4e, with conditions for maximum resolution (light region) indicated by the position of the cross-hairs (53°C, t_G = 27 min).

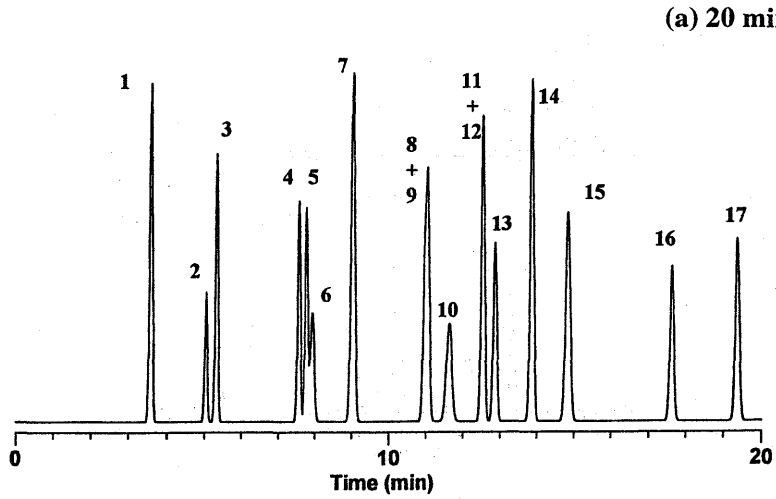


Figure 4. Method development for a 17-component sample according to plan of Figure 2b. Computer simulations based on data reported in [12,16]. Sample: a mixture of 8 substituted benzoic acids plus 9 substituted anilines: 1) 4-methoxyaniline; 2) 2,3-dimethylmethylaniline; 3) *N*-ethylaniline; 4) 3,5-dimethylaniline; 5) phthalic acid; 6) 4-chloroaniline; 7) 2-nitrobenzoic acid; 8) 3-cyanobenzoic acid; 9) 2-fluorobenzoic acid; 10) 3-chloroaniline; 11) 2-chlorobenzoic acid; 12) 3-nitrobenzoic acid; 13) 3-fluorobenzoic acid; 14) 2,6-dimethylbenzoic acid; 15) 2-chloroaniline; 16) 3,4-dichloroaniline; 17) 3,5-dichloroaniline. Conditions: 15 × 0.46 cm C_{18} column; 5–65% B gradients unless noted otherwise (as in g); A-solvent, citrate buffer, pH 2.6; B-solvent, acetonitrile; 2.0 ml min^{-1} (a–d, f) gradient time and temperature noted; (e) resolution map obtained from experiments (a–d); (g) gradient from 10–33 %B for indicated time and temperature (see figures b – g on next pages)

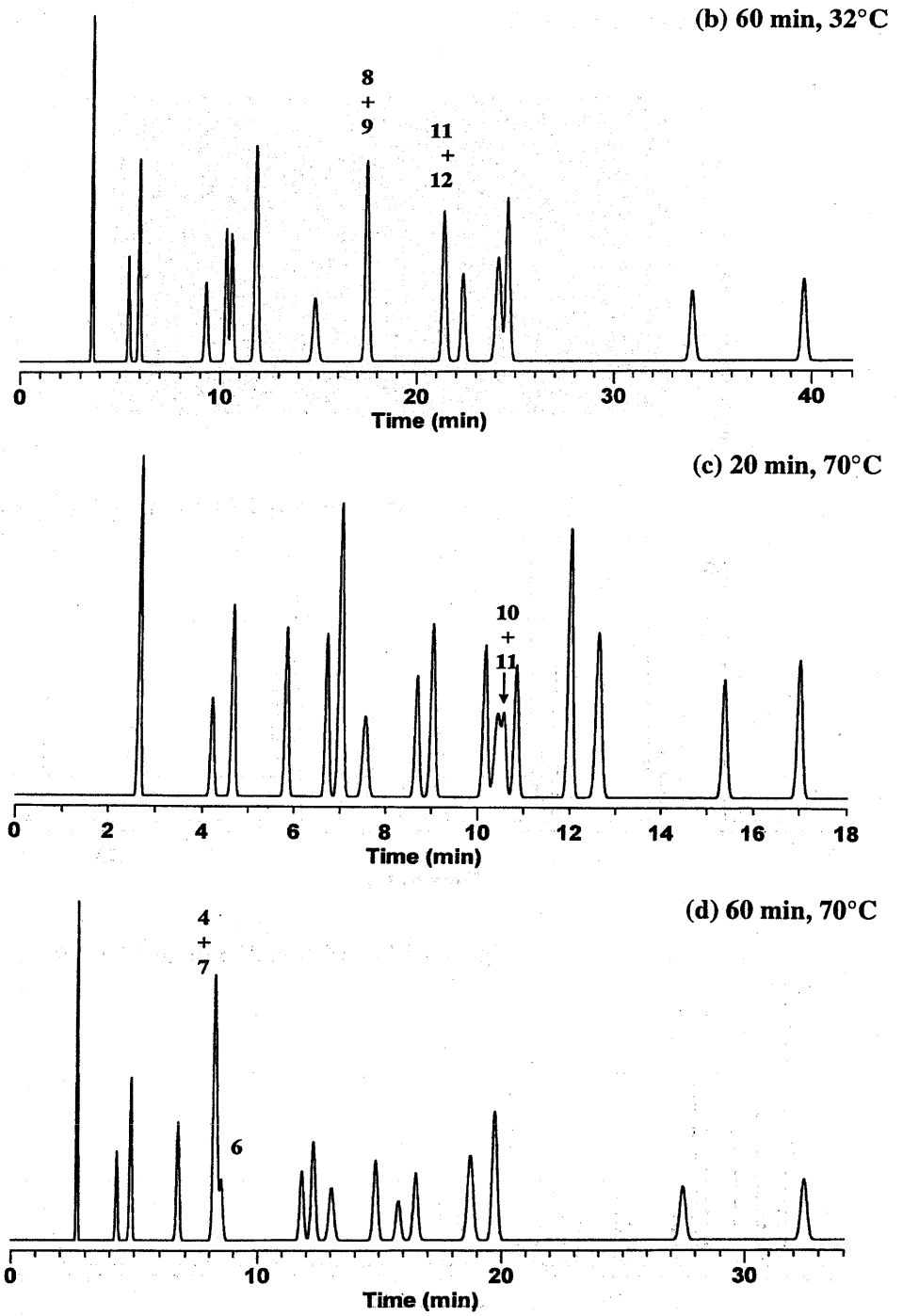
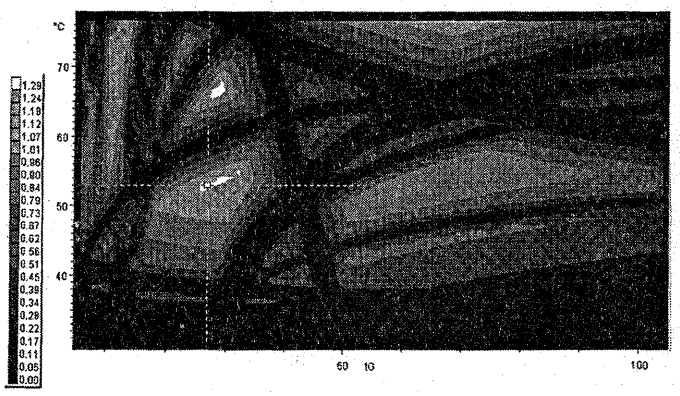
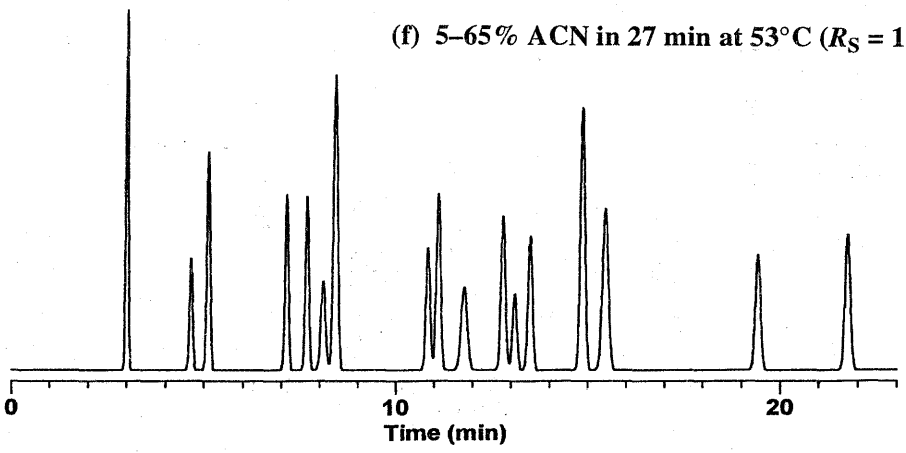


Figure 4. (continuation)

(e) resolution map



(f) 5–65% ACN in 27 min at 53°C (R_S = 1.3)



(g) 10–33% ACN in 37 min at 71°C (R_S = 1.9)

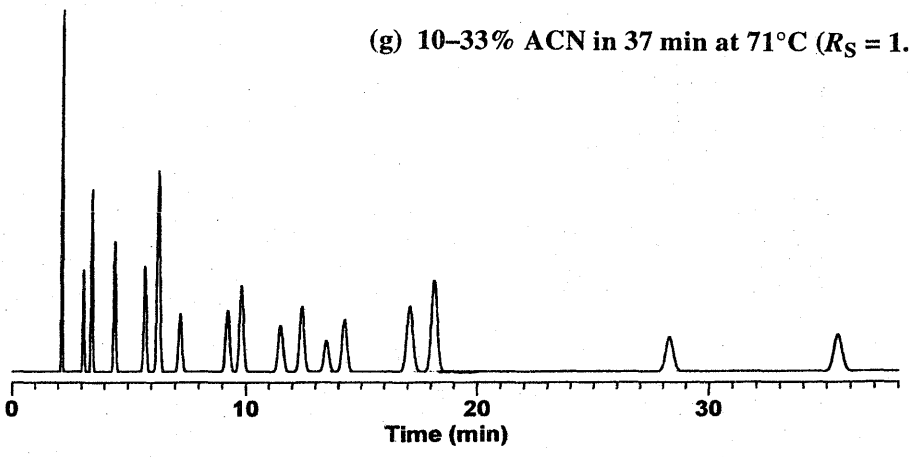


Figure 4. (continuation)

The resulting separation, for which R_S=1.3, is shown in Figure 4f. It is often possible to improve on an "optimum" separation as in Figure 4f by further change in the gradient. If the initial %B value for the gradient is allowed to vary, and if temperature and gradient steepness are re-optimized for each value of initial %B, then it is possible to increase resolution further [14]. The complete optimization of a linear gradient in this way, which would require several thousand trial-and-error computer simulations, can be achieved automatically by selecting the "Optimum Linear Gradient" option provided by the DryLab software (in a computer calculation time of 10–60 sec). The result of such an optimization for the present sample is shown in Figure 4g. In this case, the improvement in resolution (R_S = 1.9 *vs.* 1.3 in Fig. 4f) is substantial. However, increases in resolution of this magnitude (1.5-fold) as a result of optimizing initial %B are unusual. A more typical increase in R_S is 10–20% [14], but this is worthwhile since run time is often shortened at the same time. The largest changes in sample resolution as a function of initial %B will occur when critical bands elute near the beginning of the chromatogram.

Variation of temperature and isocratic solvent strength (%B)

Figure 2b illustrates a third option for initial method development, based on the variation of temperature and isocratic % organic. This option is also available starting with the same experiments used for Figure 2c, because gradient experiments can be

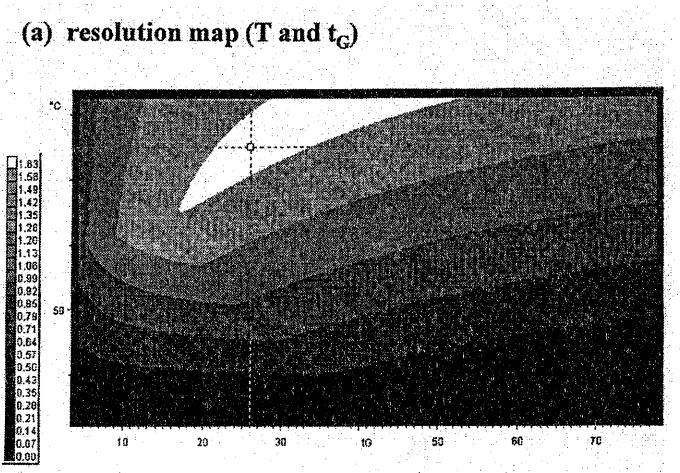


Figure 5. Method development for a sample containing eight substituted benzoic acids, based on plan of Figures 2b and c. Computer simulations based on data reported in [12,16]; see text for details. Sample: 1) phthalic acid; 2) 2-nitrobenzoic acid; 3) 3-cyanobenzoic acid; 4) 2-fluorobenzoic acid; 5) 2-chlorobenzoic acid; 6) 3-nitrobenzoic acid; 7) 3-fluorobenzoic acid; 8) 2,6-dimethylbenzoic acid. Conditions: 15 × 0.46 cm C_{18} column; 5–50% B gradients unless noted otherwise; A-solvent, citrate buffer, pH 2.6; B-solvent, acetonitrile; 2.0 ml min^{-1} (a) resolution map for gradient separations as a function of temperature and gradient time t_G; (b) predicted chromatogram for optimized conditions of 75°C and t_G = 26 min; (c) resolution map for isocratic separation at 75°C as a function on of %B; (d) predicted chromatogram for optimized isocratic conditions of 75°C and 12%B; (e) same as (d), except 5.0 ml min^{-1} (see figures b – e on next page)

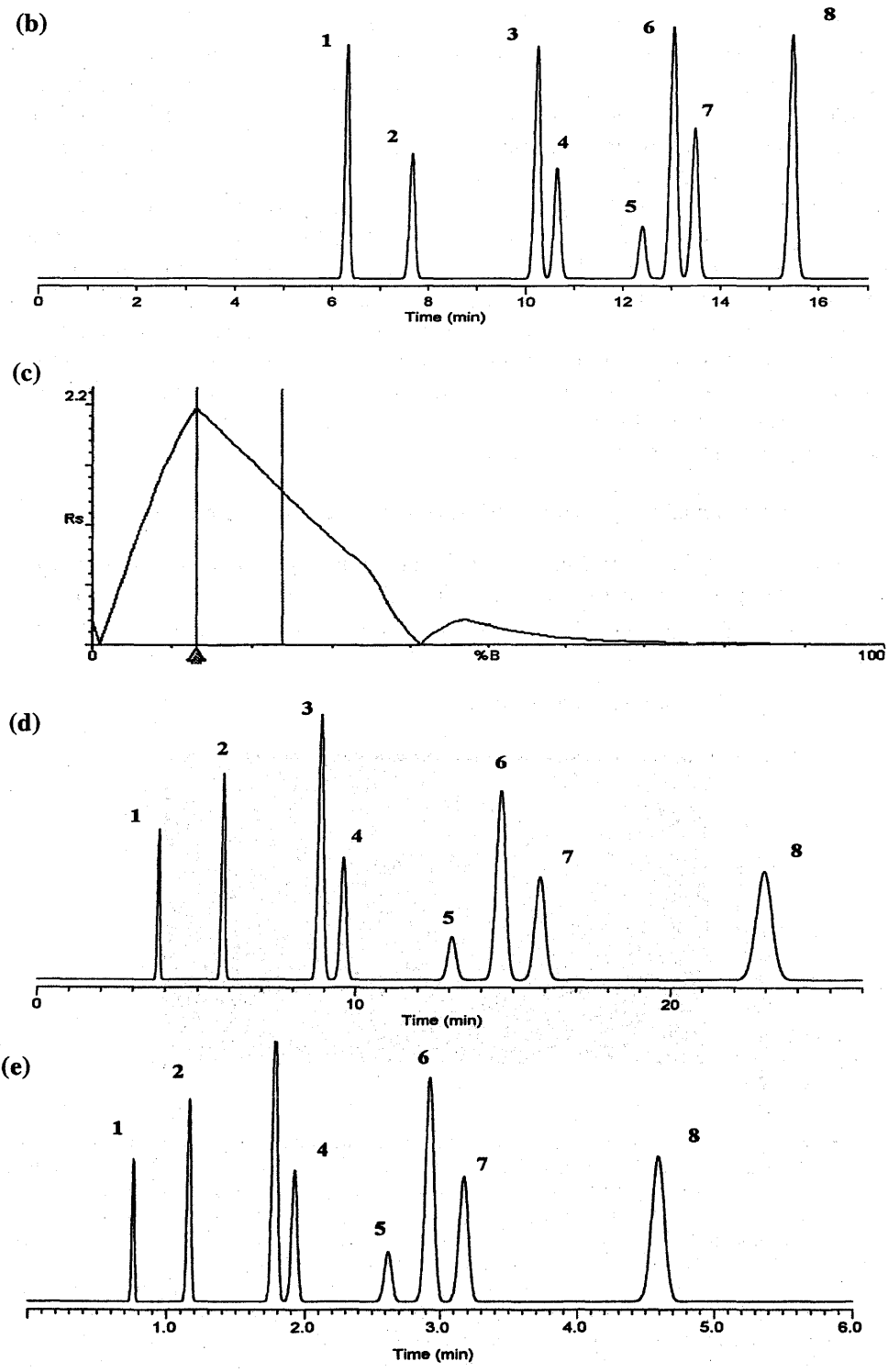


Figure 5. (continuation)

used to predict isocratic retention. As example, we will use a sample composed of eight substituted benzoic acids which can be separated isocratically, and we will begin with gradient experiments as in Figure 2c. The resolution map which plots R_s *vs.* temperature and gradient time is shown in Figure 5a. Maximum resolution occurs for 75°C and a 26 min gradient, with the resulting chromatogram shown in Fig. 5b. Resolution is adequate (R_s = 1.6), but we would normally prefer an isocratic separation, if possible.

Examination of Figure 5b shows that the retention time difference for the last and first peaks is (15–6) = 9 min. The gradient time is 5–50% in 26 min, corresponding to a 0–100% gradient in (100/45) × 26 = 58 min. If the retention time difference (9 min in this example) is less than 1/4 the gradient time for a 0–100%B gradient (58 min), then an isocratic separation is possible ([1] chapter 8).

This is the case for the present sample (9/58 = 16%). Because of the essential similarity of isocratic and gradient elution when the sample bands have similar retention (as when isocratic elution is feasible), the optimum temperature will be similar for both isocratic and gradient elution. Therefore, we can select T = 75°C on the basis of Figure 5a and use DryLab to predict separations for two different gradient times (20 and 60 min). These separations can be transferred into a new DryLab sample file and used to predict isocratic separations at 75°C as a function of %B (% ACN in the present example). The resulting resolution map for R_s as a function of %B is shown in Figure 5c. Maximum resolution (R_s = 2.1) occurs for 12% ACN, and the resulting optimized separation is shown in Figure 5d.

ADJUSTING COLUMN CONDITIONS (N)

Following the selection of conditions for "optimized" band spacing and maximum sample resolution, the separation at this stage may either (a) require a further increase in resolution or (b) a reduction in run time. Such changes in separation can be achieved in most cases by a change in "column conditions": column length, column-packing particle size or flow rate [1]. For a significant increase in resolution (by 50% or more), it is usually best to increase column length and reduce flow rate. Conversely, for a shorter run time (by a factor of two or more), one can reduce column length and increase flowrate.

The DryLab software allows the user to change column conditions and to predict the resulting separation (no additional experimental runs required). This procedure is illustrated in the example of Figure 3, where R_s=1.3 after optimizing mobile phase composition (Fig. 3h). In as much as a resolution of R_s > 1.5 is usually desired [1], a change in column conditions is suggested. In this case, an increase in column length from 15 cm in (h) to 25 cm in (i), accompanied by a reduction in flow rate from 2.0 ml min^{-1} in (h) to 1.0 ml min^{-1} in (i), results in an increase in resolution to R_s = 2.2 in (i). Note that it is often necessary to reduce flow rate when increasing column length; otherwise, the resulting increase in column pressure may be unacceptable. A column pressure < 2000 psi is preferred, if possible [1].

When there is an excess of resolution (R_s>>1.5), it is possible to shorten run time by a large factor (>2-fold) by a decrease in column length with an increase in flow rate. As an example, consider the separation of Figure 5d. The resolution of this separation (R_s = 2.1) is more than required, and the column pressure is low (300 psi) for this 15 cm column and 1.0 ml min^{-1} flow rate. Because resolution does not decrease very much as flow rate is increased, this suggests a higher flow rate to shorten run time without compromising the separation. Figure 5e shows the computer prediction for a 5-fold increase in flow rate (5.0 ml min^{-1}), and a comparable shortening of the run time (to 5 min). The resolution is adequate (R_s=1.9), with an acceptable column pressure (1500 psi).

A preferred particle size is usually selected prior to the first experiments in method development. The general rule for choosing particle size is that smaller particles give higher resolution and/or shorter run times, and for this reason smaller particles are preferred. However, particles smaller than 5 μm can give rise to certain problems. Because these smaller particles are used in shorter column lengths, the effects of instrumental (extra-column) band broadening will be greater. More serious is the fact that smaller-particle columns are more likely to plug, due to obstruction of the inlet frit by particulates from the sample, mobile phase or equipment wear. Recent works [14,15] have shown that 3.5 μm particles can be packed with 2.0 μm frits so as to eliminate column plugging as a significant problem. An increasing number of laboratories have therefore replaced the traditional 5 μm columns with these newer 3.5 μm columns.

COMPUTER SOFTWARE FOR HPLC METHOD DEVELOPMENT

The preceding discussion has emphasized the use of computer simulation, using the DryLab computer program. A number of other method development software packages have been described in the literature [1,8,17]. These various programs can be divided into two groups: (a) software which uses experimental data to derive parameters such as S and k_w that can be used to predict separation, and (b) software which bases predictions of separation on the molecular structure of compounds in the sample. Whereas programs of the first kind are generally quite reliable in predicting separation for a change in conditions, programs of the second kind are not. Only crude estimates of retention can be made on the basis of molecular structure, and it is questionable whether such estimates are of much value to the chromatographer. Software of the first kind usually relies on various theoretical and empirical models of proven reliability, and in this way it is also possible to predict separation as a function of both column and gradient conditions. The DryLab software has been evaluated extensively [1,8,12,13,18,19 and review of 20] and found to be adequately accurate for use in method development. For a further discussion of method development software, see [17].

CONCLUSIONS

Since the beginning of HPLC in the late 1960s, the question of how method development should best be pursued has been the subject of countless research projects and publications. The present discussion will not end this search for a "best" method development procedure. Nevertheless, the chromatographer can now select a method development approach on the basis of the requirements for a given sample and what is known about separation *vs.* experimental conditions. A good starting point is provided by a review of the literature prior to 1997, as set forth in [1]. Subsequent works [5,13,16,18,19] have further confirmed the value of temperature and either %B or gradient time as variables that are effective in controlling band spacing and maximizing resolution, especially in combination (as in the method development procedures summarized in Figs. 2b and c).

While we have not discussed sample composition as a factor in HPLC method development, general rules are now available [1] for anticipating necessary changes in conditions for different kinds of samples; *e.g.*, high- *vs.* low-molecular mass analytes, samples of biological origin (peptides, proteins, oligonucleotides, *etc.*), achiral or chiral isomers, *etc.* It is also known when a particular variable (*e.g.*, temperature) is less likely to change band spacing for a given sample, and what alternatives might better be pursued [1,5]. In short, the information needed for effective HPLC development is now available to the chromatographer. It remains to use (and further evaluate) what we know in this respect. The use of computer simulation in method development seems likely to increase with the passage of time, because this capability almost always leads to better methods with less effort.

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Glossary of terms

ACN	acetonitrile
k	solute retention factor in isocratic elution (same as k')
k^*	effective retention factor in gradient elution (Eqn. 2)
k_w	value of k for $\phi = 0$ (water as mobile phase; Eqn. 3)
MeOH	methanol
N	column plate number
R_s	resolution of two adjacent bands
S	solute parameter equal to $d(\log k)/d\phi$ (Eqn. 3)
t_G	gradient time (min)
t_0	column dead-time (min)
THF	tetrahydrofuran
α	separation factor for two adjacent bands (also referred to as "selectivity" or "band spacing")
ϕ	volume fraction of B solvent in the mobile phase (B is an organic solvent); equal to 0.01 %B
$\Delta\phi$	change in ϕ from beginning to end of a gradient

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