ESSENTIAL GUIDES TO METHOD DEVELOPMENT IN LIQUID CHROMATOGRAPHY

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Introduction: Steps in Method Development

Development of a method for a high performance liquid chromatography (HPLC) separation can be a major undertaking. Before the separation can be made, the sample must be in a suitable form to inject, and pretreatment steps are often required to remove major interferences or materials that might shorten the column life. After conditions for adequate separation are determined, some level of method validation is usually performed. Sample pretreatment and method validation are beyond the scope of the present discussion, which concentrates on achieving separation. This article describes only the major steps that are required for most samples. For additional information, the reader is urged to consult the reference by Snyder *et al*. (see Further Reading) which covers HPLC method development in detail. Additional method development information can be found in the other monographs listed.

General Approach

There are different approaches to HPLC method development, but we will follow the steps outlined in **Table 1** and discussed below. For most samples, this approach provides the highest probability of success with the minimum investment in time and effort.

The first step in HPLC method development is to choose a chromatographic mode or method type. The most common modes are reversed-phase, normal-phase, ion exchange and size exclusion. User surveys over the last 10 years consistently show that most separations are performed using reversed-phase columns. For the present discussion, reversed-phase separation is assumed. The following section gives a brief description of the use of alternative HPLC modes for special samples.

Table 1 General approach to HPLC method development

Select HPLC method
Obtain minimal separation
Check for and correct peak shape and width problems
Fine-tune primary variable
Change additional variables
Adjust column conditions

Once a mode is selected, the next step is to find conditions that will provide a separation of most of the sample components. When this has been achieved, it is then possible to estimate the effort that will be required to obtain an adequate separation of all components. This first step can be accomplished using either gradient or isocratic elution. We favour an initial gradient run, because all peaks are likely to elute in a defined time with reasonable separation of both early and late peaks. Usually several isocratic runs are required to achieve a similar result, and often no isocratic conditions will provide an acceptable separation. From the initial gradient run it is possible to estimate whether isocratic elution is possible. If this is the case, it is also possible to estimate conditions that give reasonable separation of most sample components.

As soon as this minimal separation is obtained, the chromatogram should be examined for problems related to peak shape. Most obvious are peak tailing problems. Although perfectly symmetrical peaks are preferred, many separations (usually for samples that contain basic compounds) will have one or more peaks that exhibit tailing. Most workers will accept peaks with asymmetry factors, $A_s \leq 2.0$ (United States Pharmacopeia (USP) tailing factor, $T_f \leq 1.7$). More severe tailing suggests the presence of unwanted sample interactions with the stationary phase. The most common fixes for tailing bands, in order of decreasing usefulness, are:

1. the use of columns designed for the separation of basic samples (based on very pure, type B silica);
2. adjustment of pH;
3. addition of triethylamine as a tailing suppressor;
4. use of ion pairing;
5. switching to a nonsilica (e.g. polymeric) column.

Symmetrical peaks that are too broad can also signal poor chromatographic behaviour; e.g. when column plate numbers, N, for the sample are $< 60\%$ of the column manufacturer's test report. Broad peaks can result from the use of too strong a sample solvent, injection volumes that are too large, column overload or column problems. Usually it is advisable

first to repeat the separation on a new column, to be sure that the problem is caused by a bad column. Reducing the injection volume to $< 25\ \mu L$, keeping the injected mass $< 10\ \mu g$, matching the injection solvent with the mobile phase and increasing the column temperature are some possible approaches to sharpening broad peaks.

Once acceptable peak shape is obtained, the next step is to fine-tune the primary variable: the percentage of organic solvent in the mobile phase, $\%B$, for isocratic separations, or gradient time, t_G, for gradient elution. In general, weaker (lower $\%B$) isocratic mobile phases of shallower (larger t_G) gradients will increase resolution at the expense of longer run times and broader peaks (with lower detection sensitivity). The best separation depends on the relative importance of peak resolution, run time and detection sensitivity, and will usually correspond to an intermediate value of $\%B$ or t_G.

An example of the effect of isocratic solvent strength ($\%B$) on retention and selectivity is seen in **Figure 1** for the simulated separations of eight aromatic compounds. It is seen that retention and bandwidth increase inversely with $\%B$. In general, R_s also increases, but not for every peak pair – only seven out of the eight peaks are visible at 70% and 50%B. Note the relative forward movement of benzene from 70%B, where it co-elutes with 2-nitrotoluene to 50%B, where it co-elutes with 2,6-dinitrotoluene; at intermediate solvent strengths it is resolved from neighbouring peaks.

Figure 2 shows the effect of gradient time (t_G) on retention and selectivity for simulated separations of a proprietary mixture of 11 herbicides. Retention and bandwidth increase with increasing t_G. The overall resolution increases with longer gradients, but note that peak 7, which elutes after peak 6 in the 20 min gradient, moves ahead of peak 7 with longer gradient times.

When satisfactory separation cannot be obtained by adjustment of the primary variable ($\%B$ or t_G), the usual problem is one of overlapping bands or selectivity. In the latter case, other conditions (mobile phase, column packing, temperature) can be varied. For example, we recommend starting with acetonitrile as the B solvent. Changes in selectivity are often observed if methanol or tetrahydrofuran is used instead of acetonitrile. Other variables worth examining are column temperature, pH (for ionic samples), use of ion-pairing reagents (ionic samples) and different types of stationary phase (e.g. change from C_{18} to a cyano or phenyl phase).

The final step in method development is to adjust so-called column conditions: flow rate, column dimensions and/or packing particle size. Typically, sample resolution increases only slowly with decrease in flow rate or increase in column length, while run time increases much faster. If resolution is greater than required, this means that an increase in flow rate and/or decrease in column length can be used for a significant decrease in run time with acceptable loss in resolution. Smaller particle columns are typically used in shorter lengths; these small particle columns can provide shorter run times without loss in resolution or increase in column pressure. The column pressure drop (or system pressure) increases with higher flow rates, longer columns and smaller particles. Since it is desirable to maintain a system pressure < 200 atm, this places a further constraint on the latter column conditions.

The simulated chromatograms of **Figure 3** show the effect of changes in column conditions on the aromatic sample of Figure 1. The lower run is the same as the middle run of Figure 1, using a 250 mm, 5 μm particle column with a flow rate of 2 mL min^{-1}, generating $R_s = 2.0$ in 11 min with 100 bar back pressure. By changing to a 150 mm, 3.5 μm column at the same flow rate, the run time is reduced to 6 min. For many applications, the narrower peaks (and thus lower detection limits) and shorter run time will be worth the minimal loss in resolution and increase in pressure ($R_s = 1.85$, 120 bar back pressure). If lower resolution is acceptable, a shorter column (75 mm, 3.5 μm) at a higher flow rate (4 mL min^{-1}) will reduce the run time to < 2 min, as shown in Figure 3C ($R_s = 1.15$, 120 bar back pressure).

Choice of HPLC Mode

Reversed-phase HPLC will prove adequate for most samples. Sample types requiring other chromatographic methods are summarized in **Table 2**. For samples that fall in one of these categories, consult the Further Reading section for detailed instructions.

Choice of Starting Conditions

A recommended set of starting conditions is summarized in **Table 3**. A C_8 or C_{18} column is chosen, with no particular preference for either phase. The 150×4.6 mm column size packed with 5 μm particles is capable of achieving most separations; with flow rates of 1–2 mL min^{-1}, run times are usually < 15 min. One of the newer type B (low metal) silicas is strongly recommended for optimum peak shape and better column-to-column reproducibility.

Acetonitrile–water is recommended as mobile phase, because of its lower viscosity (and lower pressure drop), as well as its ability to be used with low

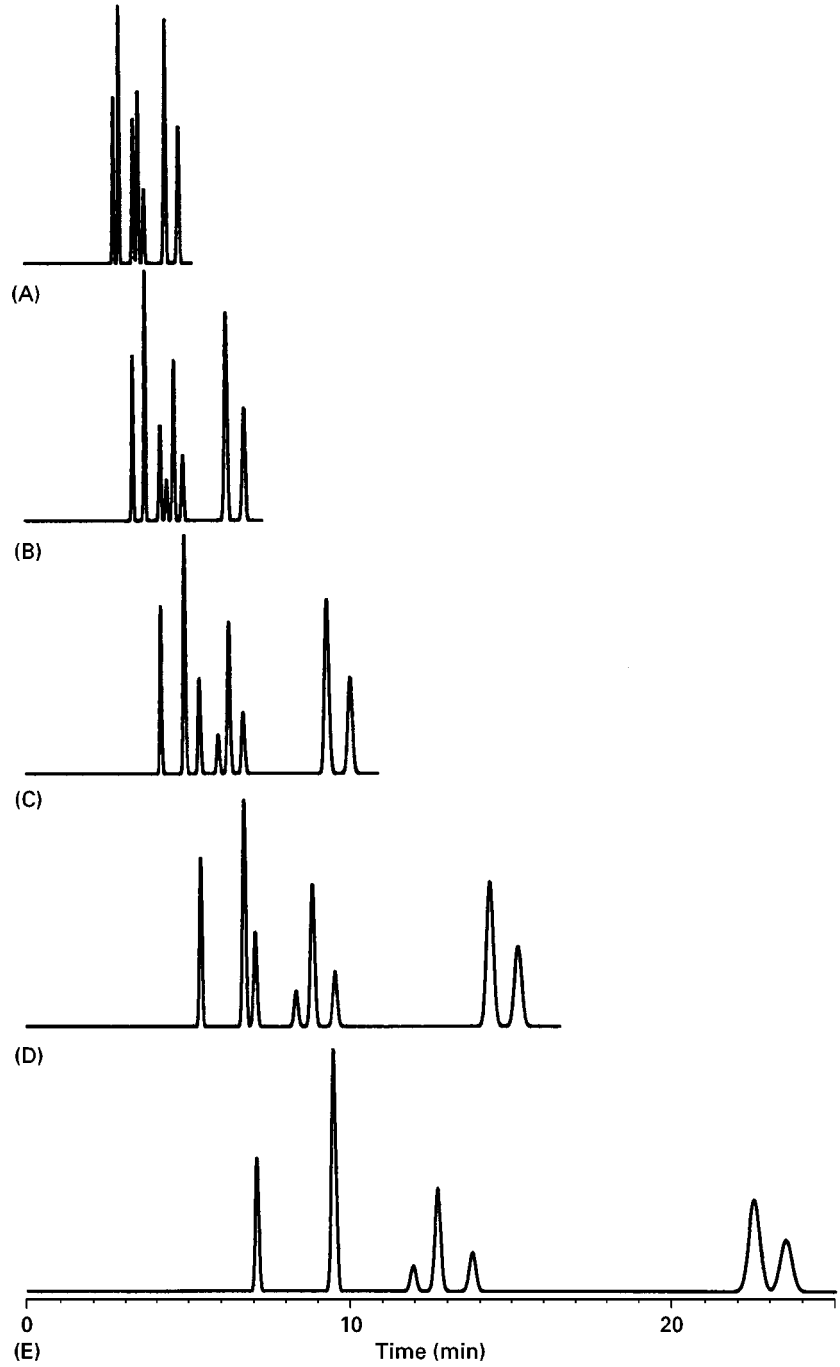

Figure 1 Simulated chromatograms for isocratic separations of aromatic compounds on a C_{18} column using water–acetonitrile mobile phases. (A) 70%; (B) 65%; (C) 60%; (D) 55%; (E) 50% acetonitrile. Samples: nitrobenzene, 2,6-dinitrotoluene, benzene, 2-nitrotoluene, 4-nitrotoluene, 3-nitrotoluene, 2-nitro-1,3-xylene, and 4-nitro-1,2-xylene (in retention order).

wavelength UV detection (≥ 190 nm; required for assay of some samples). If ionizable compounds are present in the sample, a buffer should be used. Phosphate at pH 2.5 is recommended for the initial separation, but note its reduced solubility for $> 80\%$ acetonitrile–buffer. When a volatile buffer is needed for liquid chromatography–mass spectrometry (LC-MS) applications, 0.1% trifluoroacetic acid (pH 1.9), formic acid or ammonium acetate can be used.

The column should be thermostatted to maintain constant temperature and retention times; 5–15°C above room temperature is recommended for the initial separation. Temperature can be further adjusted to change selectivity if necessary. A sufficient

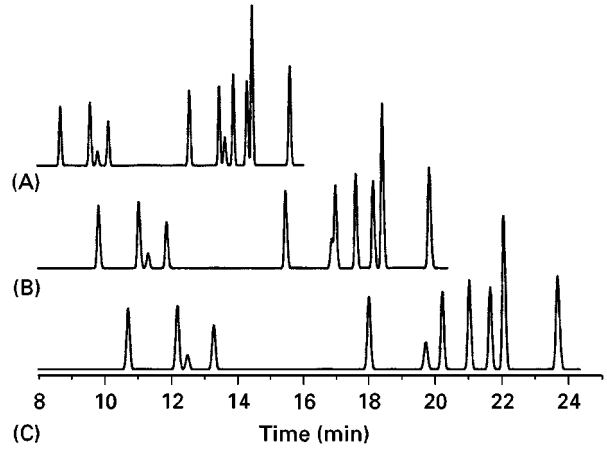

Figure 2 Simulated chromatograms for gradient separations of a proprietary herbicide mixture with a buffer–acetonitrile mobile phase. 5–80% acetonitrile in (A) 20; (B) 30; (C) 40 min.

weight of sample must be injected to obtain adequate detection sensitivity, but weights > 10 μg should be avoided initially. Similarly, sample volume should be < 50 μL to avoid excess band broadening.

Control of the Separation: Selection of Conditions

The selection of conditions for an HPLC method is expedited by a systematic approach. Because the goal of most separation development is to establish resolution for some or all peaks in a chromatographic run, we will use the fundamental resolution equation (eqn [1]) as a guideline:

$$R_s = \tfrac{1}{4}(N^{0.5})(\alpha - 1)(k/(1 + k)) \qquad [1]$$

i ii iii

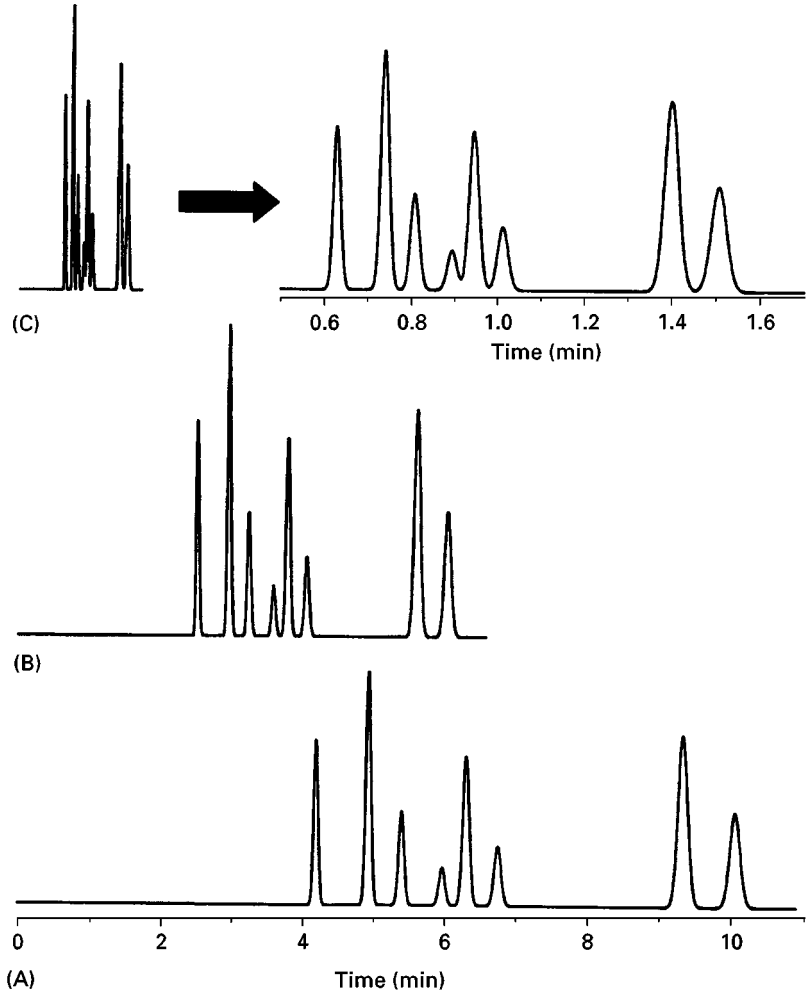

Figure 3 Simulated chromatograms for the sample shown in Figure 1 (60% acetonitrile) when column conditions are varied. (A) 250 mm, 5 μm particle column at a flow rate of 2 mL min^{1}; (B) 150 mm, 3.5 μm at 2 mL min^{1}; (C) 75 mm, 3.5 μm at 4 mL min^{1} (expanded scale in upper right).

Table 2 Preferred HPLC methods and columns for different samples

Sample characteristics	*Preferred HPLC method/column*
High molecular weight	Special columns usually required Size exclusion and ion exchange HPLC often preferred
Optical isomers (enantiomers) present	Special chiral columns required
Other isomers (stereo-, position, etc.)	Normal-phase often best, especially with unmodified silica
Mixtures of inorganic salts	Ion chromatography
Carbohydrates	Amino-bonded phase columns with reversed-phase conditions; ion exchange resins
Biological samples	Special conditions often required for life science samples; may not require different approach
Hydrocarbon mixtures	Normal-phase with unmodified silica

where R_s is the resolution, N is the column plate number, α is the separation factor (selectivity), and k is the retention factor. The influence of each of these variables on the separation is discussed below.

Control of Retention

Term iii of eqn [1] varies with solvent strength. For reversed-phase separations, increased $\%B$ increases solvent strength, reduces sample retention (values of k), and reduces the size of term iii (and the value of R_s). The retention factor, k, is calculated using eqn [2]:

$$k = (t_R - t_0)/t_0 \qquad [2]$$

where t_R is the retention time and t_0 is the column dead time. In general, as k increases, resolution and run time increase while bandwidth increases and peak height (sensitivity) decreases. For the best chromatographic performance, separations in which $0.5 < k < 20$, or better $1 < k < 10$, are preferred. If k is too small (low retention), resolution is often poor because peaks tend to bunch at t_0, while interferences from unretained sample components can also be a problem. When k is too large, run times are excessive, and detection sensitivity suffers because of wide peaks. Because of the major effect of solvent strength on separation, the selection of an acceptable value of $\%B$ should be the first priority. As will be seen in the following section, separation selectivity may also be affected by $\%B$. Examples of the effect of $\%B$ on the separation were discussed earlier in conjunction with the chromatograms of Figure 1.

Table 3 Experimental conditions for initial isocratic HPLC separation

Separation variable	*Preferred initial choice*
Column	
Dimensions (length, i.d.)	150 × 4.6 mm
Particle size	5 μm
Stationary phase	C_8 or C_{18}
Mobile phase	
Solvents *A/B*	Water–acetonitrile
$\%B$	Variable
Buffer	25 mmol L^{-1} phosphate, pH 2.5 or 0.1% trifluoroacetic acid
Additives[a] (e.g. ion pair reagents, amines)	As necessary
Flow rate	1–2 mL min^{-1}
Temperature	40°C
Sample size	
Volume[b]	< 50 μL
Mass[b]	< 100 μg

[a]Mainly affecting separation of ionized compounds.
[b]Assumes 150 × 4.6 mm reversed-phase column.

For isocratic method development, the rule of three can be used as a guideline to adjust retention by varying $\%B$. The rule of three states that retention (or k) changes about threefold for a 10% change in mobile-phase $\%B$. Thus, a change from 50% methanol to 60% methanol will reduce retention times by about three times. Similarly, a 20%B change will cause a 3×3 or about 10-fold change in retention. A convenient way to select a value of $\%B$ for isocratic separations is to start at 90% or 100% B and reduce $\%B$ in 10% steps until retention is in a reasonable range, then carry out final small adjustments in this variable.

Control of Selectivity

The selectivity term, ii, of eqn [1] is based on the separation factor α, defined in eqn [3]:

$$\alpha = k_2/k_1 \qquad [3]$$

where k_1 and k_2 are values of k for the first and second peaks of interest, respectively. Because α is related to k, changing k by varying $\%B$ often results in changes in selectivity as well. Because the optimization of term iii of eqn [1] depends on the adjustment of $\%B$, corresponding changes in selectivity (term ii) are conveniently made at the same time (by small further adjustments in $\%B$). Note that acceptable values of

term iii can often be achieved by any value of $\%B$ within a 5–10% range. The ease of changing $\%B$ is a further reason for using this variable to vary selectivity.

Although selectivity is influenced by $\%B$, changes in other conditions can have a much larger effect on values of α. Changes in the organic solvent type, pH, or use of additives are usually the next choice, once mobile-phase $\%B$ is optimized in terms of both k and α. For simplicity, we recommend changes in organic solvent first for neutral compounds. After starting with acetonitrile, change next to methanol, while reserving tetrahydrofuran as a last choice for solvent type. Changes in temperature can provide further changes in selectivity, especially for acid and base samples. If the sample contains acids and/or bases, changes in mobile-phase pH can be the most powerful means to control selectivity. If none of these approaches is successful, mobile-phase additives, such as ion-pairing reagents may be helpful, or a different kind of column (C_{18}, cyano, phenyl) can be tried.

Control of Column Efficiency

We have noted that sample resolution is not much increased by changes in column conditions (except for a large increase in run time). For the same reason, the column plate number N and term i of eqn [1] usually cannot provide a large increase in resolution, once terms ii and iii have been optimized. This means that it is important to select initial conditions which provide a value of N that will be sufficient for most separations. For most samples, we recommend one of the newer columns based on a low metal silica, often termed type B or base-deactivated silica. These columns reduce unwanted chemical interactions that cause peak tailing and column-to-column variations. Columns packed with spherical 5, 3.5 or 3 μm particles are preferable. We favour either (a) 150×4.6 mm, 5 μm or (b) 75×4.6 mm, 3.5 μm columns as a starting point. Column (a) is the first choice of many users because it is robust and has sufficiently low back pressure to allow operation at 2 mL min^{-1}, resulting in short run times. Smaller particles give a better compromise of plate number versus run time (for the same column pressure drop), but are more prone to problems such as blockage by particulates in the sample or mobile phase.

If the adjustment of conditions for optimization of terms ii (α) and iii (k) in eqn [1] has been successful, not infrequently sample resolution will be greater than required. In this case, run time can often be substantially reduced by increasing flow rate while decreasing column length. The latter represents the most profitable use of term i (by change in column conditions).

Gradient Elution

Most workers prefer isocratic methods for routine use. If an isocratic separation is not feasible because of too broad a sample retention range ($0.5 < k < 20$ not possible for all peaks), gradient elution is required. Even where a final isocratic method is possible, it is still advantageous to begin the method development process with a gradient run. Thus, a single gradient run can be carried out which will provide an attractive value of term iii for every sample peak, thus avoiding problems in isocratic elution that are caused by values of $\%B$ that are too large or too small (poor resolution, long run times, wide peaks and poor detection sensitivity). With isocratic separation, several runs (and several hours) may be required to find conditions for which $0.5 < k < 20$ for the sample. In contrast, equivalent conditions for gradient elution can be determined in advance and the first run can generate a reasonable separation. Furthermore, by using method development software with gradient input runs, it is easy to convert a gradient method to an isocratic one and to evaluate the trade-offs between an isocratic and gradient final method.

Typical starting conditions for gradient elution are given in **Table 4**. In general, longer gradient times (smaller $\%B$ min^{-1} changes) will give the same results (increased resolution and run time, broader and

Table 4 Recommended gradient elution starting conditions

Separation variable	*Preferred initial choice*
Column	
Dimensions (length, i.d.)	150×4.6 mm
Particle size	5 μm
Stationary phase	C_8 or C_{18}
Mobile phase	
Solvents *A*/*B*	Water–acetonitrile
$\%B$	5–100% *B* in 20 min[a]
Buffer	25 mmol L^{-1} phosphate, pH 2.5 or 0.1% trifluoroacetic acid
Additives[b] (e.g. ion pair reagents, amines)	As necessary
Flow rate	1–2 mL min^{-1}
Temperature	40°C
Sample size	
Volume[c]	< 50 μL
Mass[b]	< 100 μg

[a]With phosphate buffers and acetonitrile, use 5–80% *B* in 15 min.
[b]Mainly affecting separation of ionized compounds.
[c]Assumes 150×4.6 mm reversed-phase column.

shorter peaks) as using a weaker mobile phase (lower %*B*) in isocratic separations. The earlier discussion of the chromatograms of Figure 2 illustrated the effect of gradient time on the separation. For more detailed instructions in the use of gradient elution, consult the Further Reading section.

Method Development Strategies

The goal for method development is to obtain a robust method with acceptable resolution and run time. The strategy to reach this goal has two components. First, selectivity variables should be chosen for testing in an order that is most likely to give a successful separation in the minimum amount of development time. Second, for robustness in routine use and method transfer, the separation conditions should be as simple as possible and avoid potentially unstable parameters.

Most neutral or ionic samples can be separated successfully with reversed-phase columns and simple binary mobile phases of water or buffer in combination with one of the three primary organic solvents – acetonitrile, methanol or tetrahydrofuran. A choice of conditions should be made by investigating the parameter most likely to succeed, then moving to the next most likely, and so forth. If a satisfactory separation cannot be obtained by single-parameter optimization, the use of (simultaneous) two-parameter optimization can be explored.

Choice of Selectivity Variables

Selectivity variables should be examined one at a time in a systematic manner. Starting conditions generally will correspond to those listed in Table 3 or 4. Systematic method development can be approached by proceeding in order through the variables listed in **Table 5**, as described below.

Optimize %*B* First adjust the mobile-phase %*B* for reasonable k values and fine-tune for selectivity, as described previously. Acetonitrile is generally the first choice for organic solvent, but methanol is an acceptable alternative. Use water as the *A* solvent for neutrals or a low pH buffer for ionics.

Table 5 Choice of selectivity variables

Optimize %*B* using acetonitrile or methanol
Solvent type (acetonitrile versus methanol)
Temperature
pH (ionics)
Column type ($C_8 = C_{18} > CN >$ amide $>$ phenyl)
Additives
Tetrahydrofuran
Separation mode

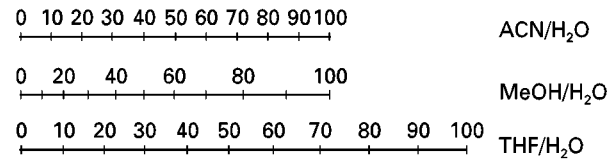

Figure 4 Solvent nomograms for reversed-phase separations. Convert percentage acetonitrile (ACN) to percentage methanol (MeOH) or percentage tetrahydrofuran (THF) by moving vertically between scales.

Solvent type If a separation cannot be obtained with acetonitrile, change to methanol and repeat the optimization experiments. A mobile phase that gives approximately the same retention time can be selected with the help of the nomogram shown in **Figure 4**. For example, 60% acetonitrile–water is roughly equivalent to 70% methanol–water. If neither solvent provides satisfactory separations, some workers will try tetrahydrofuran at this point, but we recommend delaying the use of this solvent until later. Tetrahydrofuran readily forms peroxides and is less desirable for other reasons.

Figure 5 illustrates both the use of the nomogram of Figure 4 and the selectivity that may be obtained with different organic solvents using simulated chromatograms of a 10-component steroid mixture on a C_{18} column. Figure 5A shows the best separation with acetonitrile (50%); the resolution of the critical pairs is shown. Figure 4 indicates that 50% acetonitrile is equivalent to about 60% methanol, and the methanol separation is shown in Figure 5B. The separation in methanol is significantly improved, but still unsatisfactory, so tetrahydrofuran is tried next. Figure 5C shows the 40% tetrahydrofuran separation, with baseline resolution of all peaks. Note that the nomogram is not perfect – the indicated tetrahydrofuran concentration resulted in a longer run time than for acetonitrile or methanol.

Temperature Column temperature should be controlled so that retention times do not drift. In general, a 1–2% change in retention will be observed for a 1°C change in temperature, but many workers do not appreciate that selectivity also often changes with temperature. A second experiment run at 20–30°C higher temperature will indicate if improved selectivity can be obtained by varying column temperature. While changes in α as a result of a change in temperature are smaller than for other changes in conditions, this disadvantage is offset by the greater

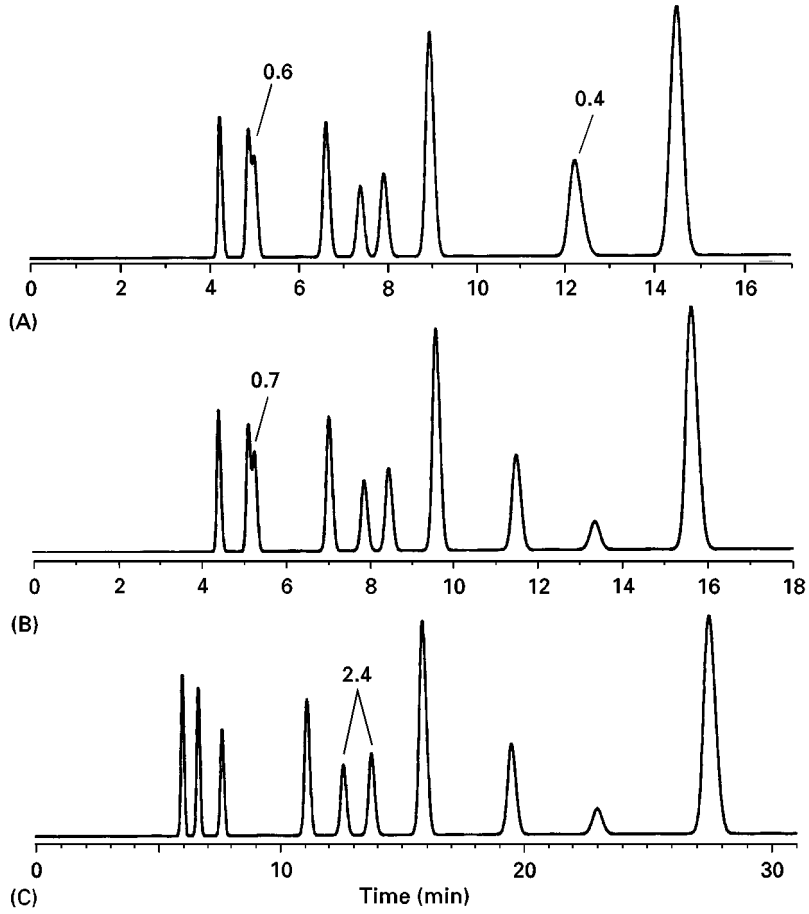

Figure 5 Comparison of selectivity changes with different organic solvents using an 11-component steroid sample and a C_{18} column (simulated chromatograms); resolution of critical peak pair(s) shown as call-outs. (A) 50% acetonitrile; (B) 60% methanol; (C) 40% tetrahydrofuran.

convenience of a change in temperature, without any offsetting disadvantages.

Mobile-phase pH If the sample contains ionizable compounds (acids or bases), mobile-phase pH represents a powerful variable for changing selectivity. Thus, values of k for ionized species will generally be much smaller than for the nonionized compound. As a result, both absolute and relative retention for acids or bases can change dramatically with small changes in pH, when the pK_a value of the compound is within 1–1.5 units of the mobile-phase pH. Be sure to use buffers within their effective buffering range (± 1 unit from the buffer pK_a). When optimizing pH, changes in steps no more than about 0.5 pH units are recommended. Note that silica-based columns are generally limited for use at $2 < pH < 8$.

On the other hand, the choice of a $pH < 3$ is advantageous for several reasons: first, the pK_a values of both acids and bases will differ from the mobile phase pH by > 2 units, so that sample retention will not vary with small changes in pH; i.e., the method will be more robust. Second, bases are usually best separated at low pH, because undesirable interactions between sample molecules and the column packing (i.e. silanols) are suppressed, thereby minimizing peak tailing and maximizing column plate numbers. However, the choice of a $pH < 3$ means that very little change in selectivity can be expected as a result of intentional changes in pH (e.g. for $2 < pH < 3$).

Column type The initial separation will usually be done on a C_8 or C_{18} column. For changes in selectivity, it is seldom fruitful to change the bonded-phase chain length (e.g. C_8 to C_{18} or C_4). Similarly, although changes in selectivity may be observed with the same phase obtained from different manufacturers, the magnitude of such changes is generally small. Rather, if the column is to be changed in order to change selectivity, it is recommended to change

to a stationary phase with significantly different chemistry.

After a C_8 or C_{18} column has been tried, a cyano (CN) phase is usually the next choice. Because cyano columns are more polar, similar retention requires the use of 10–20% less organic solvent; e.g. similar retention might be obtained with 35%*B* on a cyano column as with 50%*B* on a C_8 column.

A phenyl column is usually the next choice, if a cyano phase does not work. However, recently developed columns with an amide or carbamate function (e.g. Symmetry Shield, Zorbax Bonus RP or Discovery Amide) have proven to have unique selectivity that is also worth exploring when examining column-type effects. Additional information regarding column selection and chemistry can be found in the Further Reading section.

Additives Mobile-phase additives (in addition to buffers) can be used to enhance selectivity with some sample types. For example, ion pairing may be used to advantage when the sample contains both acidic and basic components. While large changes in selectivity are possible by varying the concentration of an ion pair reagent, ion pairing often results in long equilibration times when changing the mobile phase, as well as other problems. Optimization of additives is beyond the scope of the present discussion.

Tetrahydrofuran Tetrahdyrofuran as the *B* solvent often gives significant selectivity changes when compared to acetonitrile or methanol. Problems related to slow equilibration, equipment memory effects, excessive UV background at low wavelengths, instability and unpleasant odour make most workers delay the use of tetrahydrofuran until it is unavoidable. In spite of these potential problems, tetrahydrofuran does have unique selectivity characteristics and will often provide separations when acetonitrile or methanol have failed. If tetrahydrofuran is to be used, use Figure 4 to select starting mobile-phase conditions based on previous experiments with acetonitrile or methanol.

Separation mode When efforts at obtaining a successful reversed-phase separation prove unsuccessful, one should consider other separation modes. Several other separation modes are shown in Table 2.

Single-Variable Optimization

Traditionally, a single variable is optimized at a time during HPLC method development. In most cases, one can proceed through the list of variables in Table 5 in order, stopping when an adequate separation is achieved. A convenient procedure is to optimize the first variable, then hold that condition constant while changing the next parameter. This sequential optimization of parameters is a straightforward approach. Once all the desired variables have been optimized, it is a good idea to make small and reasonable changes around the final conditions to check robustness. For example, change $\pm$ 3–5%*B*, $\pm$ 0.5 pH units, $\pm$ 5°C, and so forth to make sure the separation is not adversely affected by such changes.

Multi-Variable Optimization

An alternate approach to single-variable optimization is to change two or more parameters at once. Two different approaches are suggested: the method development triangle and the simultaneous optimization of solvent strength (or gradient time) with a second variable. In both of these cases, one should choose variables that change selectivity in different ways, ideally orthogonal to each other in terms of their selectivity effects.

Method development triangle The method development triangle shown in **Figure 6** is a widely used approach for selecting the optimum organic solvent or mixture of organic solvents. This is a logical next step when acetonitrile, methanol and tetrahydrofuran have been optimized individually, and the least resolved peak pairs are different for at least two solvents. Each corner of Figure 6 represents a binary-solvent mobile phase with a %*B* value (for each of these three *B* solvents) that gives acceptable isocratic

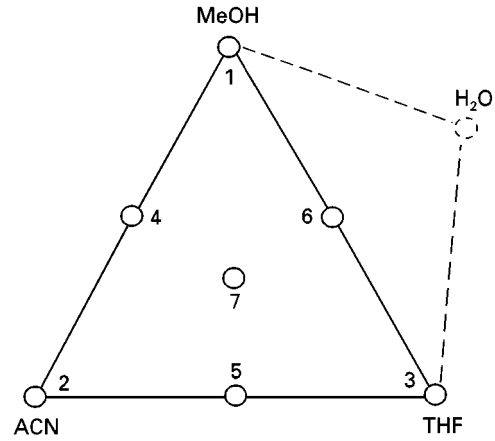

Figure 6 Solvent selectivity optimization for reversed-phase HPLC. Experiments 1, 2 and 3 are binary (water and one organic) mobile phases; 4, 5 and 6 are 1 : 1 ternary (water and two organics) blends of corner compositions, and 3; 7 is 1 : 1 : 1 quaternary (water plus three organics) blend of corner compositions.

separation; e.g. so as to give $k \approx 10$ for the last peak in each separation. These mobile phases (1, 2 and 3 in Figure 6) then are blended 1 : 1 or 1 : 1 : 1 for the remaining experiments. Once all seven experiments are run, the chromatograms can be spread out in the same grid pattern and examined for changes in selectivity between conditions. Further adjustments in solvent blends may beneficial. For simplicity and robustness, mobile phases with fewer solvents are preferred (binary > ternary > quaternary). As with other optimization strategies, the final conditions should be varied in a systematic manner to determine the robustness of the chosen mobile phase.

Two-variable optimization The method development triangle approach (shown in Figure 6) is one example of the simultaneous variation of two variables. Other two-variable optimization procedures are now possible with the recent availability of appropriate computer simulation software. This new software (DryLab version 3.0, LC Resources) facilitates the simultaneous optimization of either isocratic %*B* or gradient time and any second variable (e.g. temperature, pH, additive concentration). The combination of two variables having different selectivity actions can help identify separation conditions that are unlikely to be found using more traditional approaches. With the use of optimization software, four to six input runs allow the user to model the separation under any combination of the two variables.

An example of the results of a two-variable optimization is shown in **Figure 7** using a 150 mm C_{18} column with buffered acetonitrile to separate a 10-component mixture of benzoic acids and anilines. The best single-variable separation (at an arbitrary starting pH of 3.0) is shown in Figure 7A with $R_s > 2$, but the %*B* must be held within $\pm$ 1% and the pH within $\pm$ 0.05 units to maintain $R_s > 1.5$, so the method is not robust. Using six experimental runs and computer optimization, the lower chromatogram (Figure 7B) was obtained, offering $R_s > 2$ for $\pm$ 5%*B* and $\pm$ 0.1 pH units in half the run time.

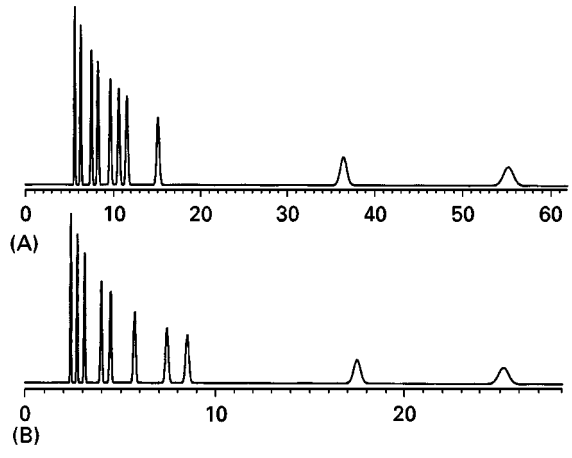

Figure 7 Simulated chromatograms for separation of 10 benzoic acids and anilines using a 150 mm, 5 μm particle C_{18} column with acetonitrile–buffer mobile phases. (A) Optimum separation at pH 3.0 (15% acetonitrile); (B) optimized %*B*-pH conditions (pH 3.4, 25% acetonitrile) using computer-assisted method development (DryLab).

Table 6 DryLab® software optimization modes

Parameter	*Input experiments*
Isocratic %*B*	2 or 3
Gradient time	2
Normal phase	3
pH	3
Ternary solvent	3
Ionic strength	3
Additive concentration	3
Temperature	2
Gradient time versus temperature	4
Isocratic %*B* or gradient time versus any variable	4–9

Computer-Assisted Method Development

Many of the above changes in separation as a function of conditions can be described in theoretical or empirical equations. The fundamental relationships defined in eqn [1] form the basis of algorithms used to predict resolution. For example, term i of eqn [1] can be calculated from first principles, term ii is defined in eqn [3], and $\log(k)$ is linearly related to %*B* (term iii). This means that two experiments differing only in %B can be used to predict resolution at any other %*B*. Similarly, basic theory can relate isocratic and gradient separations in terms of the same retention relationships. A computer program (software) can therefore be used to predict separation as a function of isocratic %*B*, gradient conditions, and/or column conditions, using two gradient runs to calibrate the sample and initial conditions. The example of Figure 7 required six runs (3 pH values at each of two gradient times) for optimization of isocratic %*B* and pH. Some of the other variables available for optimization with one of these programs (DryLab® software) are shown in **Table 6** along with the number of input experiments required. The use of optimization software is strongly recommended in order to reduce method development time, achieve more robust separations, and gain a better understanding of the separation.

Table 7 Common HPLC separation problems

Observation	*Problem source*	*Solution*
Poor peak shape	Wrong silica type	Use type B silica
	Blocked frit or column void	Replace frit, backflush column
	Silanol interactions	Use amine additives, change pH, use end-capped stationary phase
Excessive peak width	Bad column	Replace column
	Column overload	Reduce injection volume or mass
	High molecular weight	Normal
	Unresolved peaks	Improve separation
Inadequate retention	Mobile phase too strong	Use lower %*B*
	Column too weak	Switch to C_{18}
	Samples ionized	Change pH
	Samples too polar	Change to normal phase
	Gradient starting too strong	Start at lower %*B*
Excessive retention	Mobile phase too weak	Use higher %*B*
	Column too retentive	Switch to C_8, C_4 or CN
	Samples too hydrophobic	Change to normal phase
	Gradient stops too soon	Stop at higher %*B*
Excessive retention range	Acids and bases or bases and neutrals in sample	Use ion pairing
	Too broad of polarity for isocratic method	Use gradient elution
Inadequate resolution	Retention too short	Increase k
	Poor selectivity	Change α
	Plate number too low	Use longer column or smaller particle size

Troubleshooting Common Problems

Table 7 highlights some of the commonest causes of chromatographic problems likely to be encountered in the HPLC method development.

Further Reading

Dolan JW and Snyder LR (1989) *Troubleshooting HPLC Systems*. Clifton, NJ: Humana Press.

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Neue UD (1997) *HPLC Columns. Theory, Technology and Practice*. New York: Wiley-VCH.

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ESSENTIAL GUIDES TO METHOD DEVELOPMENT IN SOLID-PHASE EXTRACTION

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Solid-phase extraction (SPE) is a sample preparation technique combining nonlinear modes of chromatography for the separation, purification, concentration and/or solvent exchange of analytes of interest. SPE is the removal of chemical constituents from a flowing liquid sample via retention on a solid sorbent, and the subsequent recovery of selected constituents by elution from the sorbent. SPE was developed as an heterogeneous (two-phase) alternative to homogeneous (one-phase) liquid–liquid extraction (LLE) for the isolation of solutes from solution.