

Adjusting Conditions for a Routine Reversed-Phase HPLC Assay, Part 2: Changing Separation Conditions

Ronald E. Majors, Agilent Technologies Inc, Wilmington, Delaware, USA.

Lloyd R. Snyder, LC Resources, Orinda, California, USA.

John W. Dolan, BASi Northwest Laboratory, McMinnville, Oregon, USA.

When a column is replaced with a new or “equivalent” column, sometimes the chromatogram can change so much that it is no longer suitable for its intended use. In such instances, method adjustment is necessary to correct the change. How much can the chromatographic variables be changed before revalidation is required? What do the regulatory agencies have to say about method adjustment? The authors discuss these issues and propose a technique that can be used to speed selection of new operating conditions.

In the last instalment of “Column Watch,”¹ the problem of a possible change in column selectivity was introduced, and means for selecting a different column as a replacement were considered. Ten or twenty years ago, changes in separation were not uncommon when replacing a column with one from a different production batch. Personally, we are aware of more than a few such instances within the past decade, in some cases involving columns from major manufacturers. Although batch-to-batch column reproducibility has improved greatly in recent years,^{2,3} it is premature to claim that all columns currently manufactured will be adequately reproducible for all possible samples and separation conditions. There is also a need to improve some older methods by substituting columns of more recent design, for which the likelihood of encountering major differences in column selectivity is increased greatly.

Column substitution as described in Part 1¹ is likely to work for many cases but not for all. When a change in column does not provide acceptable separation, the only remaining option is to vary separation conditions (mobile-phase composition or temperature). This so-called *method adjustment* has been shown to be effective

generally,⁴ but it is not used routinely within the pharmaceutical industry because of questions concerning the extent of method revalidation that is required. In this article, we propose a technical review of means for method adjustment and a review of pertinent regulations concerning the required validation of adjusted methods.

Method Development versus Method Adjustment

Method development refers to the initial selection of separation conditions for a proposed assay procedure.⁵ Method adjustment involves making minor changes in separation conditions for an already developed method, usually as a means of correcting changes in separation selectivity because of a change in column. It should be emphasized that method adjustment is *not* justified for the purpose of correcting errors in mobile phase formulation, sample preparation or faulty equipment performance. An example of method adjustment is shown in Figure 1. The “original” column yielded the separation of Figure 1(a), while Figure 1(b) shows the separation after replacement of the original column with a new, nominally equivalent column from the same manufacturer.

Several changes in relative retention and separation are apparent in these two chromatograms; most notably, the baseline resolution (R_s) of peaks 4 and 5 in Figure 1(a) has degraded to partially overlapping peaks in Figure 1(b) (arrow). Arbitrary changes in conditions can be made to improve the separation of Figure 1(b), as in Figure 1(c), where the percentage of acetonitrile in the mobile phase (%B) is increased from 50 to 54.5%. However, relative retention in Figure 1(a) and 1(c) is noticeably different. “Complete” method adjustment attempts to achieve a separation that is equivalent to that using the original column, as in Figure 1(d), where both temperature and %B have been adjusted. “Equivalent” separation implies generally equivalent band spacing and resolution for each peak; small differences in absolute retention and run time can usually be minimized by further changes in flow-rate.

For a change in separation selectivity, only those variables that affect selectivity can be used in method adjustment. Temperature, gradient time, isocratic %B, pH and buffer or additive concentration are useful variables in this connection. Following the adjustment of separation selectivity, changes in flow-rate can be used to minimize differences in run time.

All of the regulatory guidelines stress the importance of system suitability tests as a measure of the ability of an analytical method to produce valid results (for example, reference 6, p. 14; reference 7, p. 10; reference 8, p. 2389). The *The United States Pharmacopoeia (USP)* (reference 8, p. 2389) states that “System suitability tests are an integral part of gas and liquid chromatographic methods. They are used to verify that the resolution and reproducibility of the chromatographic system are adequate for the analysis to be done.” As such, it should be obvious that care must be taken to select a system suitability test that indeed discriminates between a method that is operating acceptably and one that is not. The guidelines are not specific about which parameters should be included in system suitability tests. Again, a judgement call is necessary by the method development chemist. In some instances, resolution between two peaks might be the best discriminator, whereas in other situations, minimum peak area or some other criterion might also be appropriate.

An example of selection of system suitability requirements can be taken from the separations of Figure 1. This will help to illustrate the difference between “adequate” separation and “equivalent” separation. System suitability criteria should be based upon the goals of the separation. One such goal for the sample of Figure 1 might be a requirement to be able to adequately quantify each of the five peaks in the sample. One criterion could be that the peaks need to be adequately separated, with the quantitative requirement of $R_S > 2.0$ for all peak pairs. If this were the system suitability requirement, Figures 1(a), 1(c) and 1(d) would pass system suitability and would be expected to give acceptable results for real samples. Another option might be to use relative retention time as the system suitability criterion. For example, the method might require the relative retention of peak 4 to be 0.85–0.95 that of peak 5. In this instance, the separations of Figures 1(a) and 1(d) pass, but 1(c) fails. It is clear that the resolution criterion makes the method much more flexible in terms of method adjustment and would be preferred, all other things being equal. We strongly suggest that system suitability tests be designed such that “adequate” separations are acceptable [Figures 1(a), 1(c), and 1(d)] rather than requiring “equivalent” separations [Figures 1(a) and 1(d)]. The system suitability test should discriminate between method performance

that will produce acceptable versus unacceptable results, even if the separation does not look exactly the same. Selecting appropriate criteria can be a challenge.

Allowable Changes in Separation Conditions for Method Adjustment

A large change in separation conditions is essentially equivalent to method development, as opposed to “smaller” changes in method adjustment. Provisional recommendations for maximum changes in method adjustment have been proposed, as summarized in Table 1.

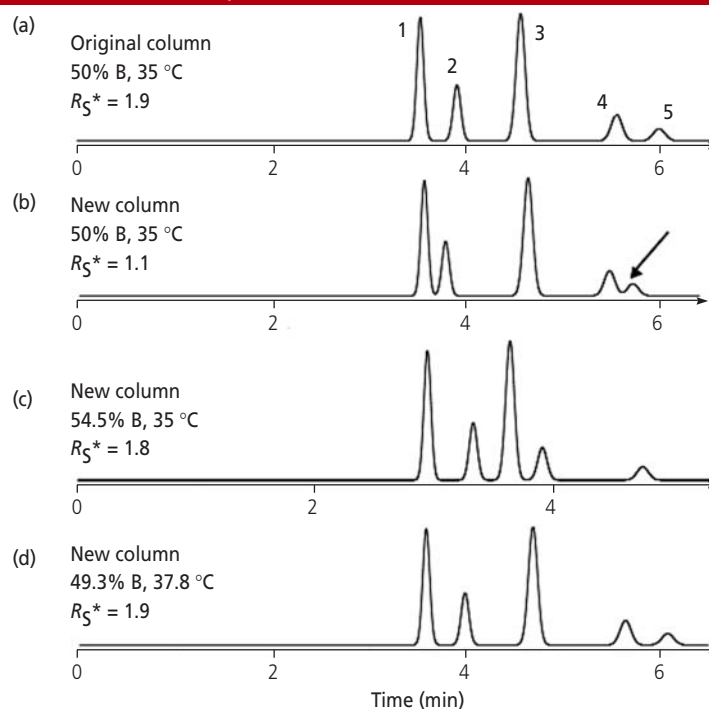
United States Food and Drug Administration (FDA) guidelines for bioanalytical (analysis of drugs in biological matrices) method validation⁶ do not directly address the requirements for revalidation following method adjustment. The guidelines imply that as long as system suitability passes for the method, it is acceptable to use. It is our opinion that modifications to method conditions that fall within the parameters of Table 1 do not require revalidation. More extensive changes, however, would likely fall under

the category of “partial validation,”⁶ for which one batch is run to confirm that the required levels of precision and accuracy are maintained under the new conditions.

The FDA guidelines for analytical procedures for drug substance (pure drug) and drug product (formulated drug)¹² are more specific than the bioanalytical guidelines.⁶ This guidance states that revalidation requirements depend upon the nature of the change. Again, system suitability is used as the criterion: “If during each use an analytical procedure can meet the established system suitability requirements only with repeated adjustments to the operating conditions... the analytical procedure should be... revalidated.”¹² Reference is made to use of compendial procedures (for example, *USP*), so it is our opinion that the adjustments of Table 1 would not require revalidation, provided system suitability passes the acceptance criteria.

International Committee on Harmonization (ICH) guidelines^{7,13} are similar to FDA guidelines.¹² These state that the revalidation requirements depend upon the nature of the change¹³ and on

Figure 1: Example of method adjustment. (a) Original column with isocratic conditions as shown, (b) new column, same conditions as in (a), (c) new column, arbitrary adjustment of conditions to restore resolution of (a), and (d) new column, conditions adjusted as described in text. Column: 150 mm × 4.6 mm C18; mobile phase: acetonitrile–buffer (pH 2.8); flow-rate: 2.0 mL/min.



Peaks: 1 = methylbenzoate, 2 = 4-*n*-pentyl aniline, 3 = *p*-nitrotoluene, 4 = 4-*n*-hexyl aniline, 5 = 4-*n*-butylbenzoic acid. Adapted from reference 4.

Column Watch

system suitability as the performance criterion;⁷ reference is made to pharmacopoeias for additional information.⁷ As such, these guidelines also imply that if the adjustments fall within the criteria of Table 1, no revalidation is required.

For any revalidation of a method subject to regulatory guidelines, a written revalidation plan should be prepared, including predetermined acceptance criteria.

The *USP* guidelines⁸ historically have relied heavily upon system suitability tests as a means of determining adequate method performance. They state, "Adjustments of operating conditions to meet system suitability requirements may be necessary,"⁸ but do not comment on revalidation requirements. More specific guidelines appear likely in the near future. *Pharmacopeial Forum* is used to propose changes to the *USP*. A recent *Forum* article¹¹ proposed the acceptance limits for parameter adjustment shown in Table 1. These are generally similar to the preceding recommendations,^{9,10} which it is likely were used as the basis for the *Forum* recommendations.

From the aforementioned review, three consistent threads are apparent. First, system suitability should be the primary criterion for the acceptability of method performance. Any change that is made such that system suitability does not pass should be a red flag for the operator. In such instances, the method should be further modified until system suitability again passes. Second, the adjustment limits of Table 1 (especially those of reference 11 when incorporated into the *USP*) generally should be acceptable for method adjustment, requiring no revalidation provided system suitability passes. Finally, the judgement of the user is very important in the decision of whether or not to re-validate the method. The guidelines of Table 1, internal company policy (standard operating procedures) and common sense should guide users in the adjustment versus revalidate decision. In any event, proper documentation should be made for any method changes.

Validation Versus Revalidation

In the context used in the present discussion, we consider validation to refer to the initial proof that the method meets the performance requirements set by users and regulatory agencies. Validation might include experiments that are not directly

related to the chromatography, such as stability of stock solutions, sample stability or extraction recovery. We refer to revalidation as the process that shows a modified method still meets the initial performance requirements. As such, repeat

of non-chromatographic experiments, such as solution stability, have no benefit and are not performed. Similarly, the number of runs required for statistical support of method performance during validation might not be necessary for revalidation.

Regulatory guidelines are not clear regarding the level of revalidation that is required, with the exception of the bioanalytical guidelines,⁶ which require, as a minimum, a single run to show that acceptable method performance can be obtained. As a result of the vagueness of the guidelines, most of the justification for revalidation (or not) lies with the user. For any revalidation of a method subject to regulatory guidelines, a written revalidation plan should be prepared, including predetermined acceptance criteria. It is expected that common sense will be used in conjunction with the guidelines of Table 1 when designing the re-validation experiments. For example, a change in mobile phase organic solvent or pH that exceeds the suggestions of Table 1 is more likely to have a negative impact on the method than a change in flow-rate or

column diameter.

Means for Efficient Method Adjustment

Method adjustment can be performed in similar fashion as for method development,⁵ but with the proviso that only "small" changes in conditions are allowed. A systematic procedure for method adjustment has been described,⁴ based upon the observation that changes in separation as a result of change in two or more conditions are additive, when the change in each condition is restricted within certain limits.¹⁴ This means that given separations for the original and new columns, only one additional experiment is needed for each condition (and either column) to optimize method adjustment. For example, if temperature and %B were the variables under consideration, data for a run at one temperature and one %B on both the original and the new column would be combined with a third run at a second temperature (with the original %B) and a fourth run with a new %B (and the original temperature) on either column. These data would be used to determine the best combination of temperature and %B to use on the new column for equivalent separation. Commercial software for performing method adjustment as described in reference 4 is available (Method Match, Rheodyne LLC, Rohnert Park, California, USA), with the result of Figure 1(d). Note that the required changes in %B (−0.7% or −1.4% relative) and temperature (+2.8 °C) for this example are well within the limits of Table 1.

The effectiveness of method adjustment as described in reference 4 was evaluated by comparing the isocratic separation of approximately 60 different samples on two nominally equivalent columns, which differed in carbon percentage by 10% (and gave significantly different separation). These samples contained 5–15 components each, and method adjustment was performed by varying temperature and %B only. The separation of each sample on the two columns was compared in terms of the coefficient of variation (CV) of resolution R_s , with a value of $CV \leq 15\%$ being regarded as "equivalent" (for example, a change in R_s from 2.0 to 1.7). Ten different samples with the same number of components were grouped, and an average CV for the group determined. Data points above the line for $CV = 15\%$ in Figure 2 correspond to possibly unacceptable separation; it is seen that all of the unadjusted separations (solid

Figure 2: Efficacy of method adjustment for approximately 60 randomly selected samples, changing only temperature and %B. CV values for differences in resolution between original and replacement. Data points for each value of n are an average of 6–10 samples each. Adapted from reference 4.

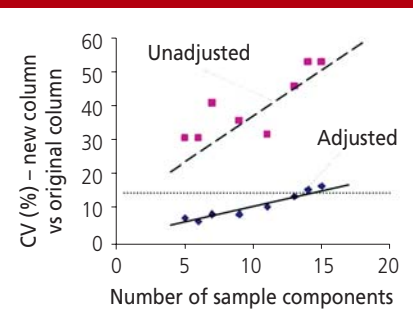


Table 1: Proposed maximum allowable changes in separation for HPLC method adjustment.

Separation Condition	Allowable Change in Condition		
	Ref. 6 (<i>PharmEuropa</i>)	Ref. 7 (<i>Pharm Tech</i>)	Ref. 14 (<i>Pharm Forum</i>)
Mobile phase pH	+1 unit (depending on pK _a of analyte)	+0.2 units	±0.5 units
Buffer concentration	+10%	+10%	±10%
Ratio of mobile phase solvents	+30% relative or +2% absolute (whichever is larger)	+30% relative or +2% absolute (whichever is larger, but no greater than +10% absolute)	+30% relative or +2% absolute (whichever is larger, but no greater than +10% absolute)
Column length	+70%	+70%	-50% to +100%
Column internal diameter	+25%	+25%	±25%
Flow-rate	+50%	+50%	±50%
Particle size	Can be reduced by 50%	Can be reduced by 50%	Can be reduced by 50%
Injection volume	+2-fold	+2-fold	Any reduction (provided acceptable precision and detection limit)
Column temperature	+40 °C	+20 °C	±10 °C
Detector wavelength		+3 nm	None, but ±3 nm allowed between detectors

squares) fail this criterion. Average values of CV are plotted in Figure 2 versus the number of components in the sample. As expected, the probability of an acceptable adjustment decreases as the number of sample components increases, with a sample containing approximately 14 components having an even chance of a successful adjustment. These results suggest that method adjustment based on change in temperature and %B will generally be successful, especially for samples containing no more than 14 components. The probability of a successful adjustment increases if additional separation conditions are varied (for example, pH or buffer concentration).

Conclusions

The challenge of obtaining adequate method performance across several years can be complicated by unexpected changes in column chemistry for nominally equivalent columns (same part number) over time. This can be further complicated when a column is discontinued or replaced with an "improved" version. In Part 1 of this series, we described a technique to select columns likely to give equivalent separations.¹ In the current discussion, we have addressed the issue of methods that are observed to change slightly when a replacement column (either of the original type or an "equivalent" column) is installed. The regulatory guidelines depend heavily upon system suitability tests as a

discriminator of method performance. In addition, there exist limits (Table 1) within which variation in chromatographic parameters are allowed by regulatory agencies (assuming the recommendations of reference 11 are adopted by the *USP*). When method changes are made that fall within the parameters of Table 1, we consider this as method adjustment, and little if any revalidation is required, so long as system suitability passes. Method changes that exceed the guidelines of Table 1 are more likely to be considered modifications of the method that will require some degree of revalidation.

References

1. L.R. Snyder and J.W. Dolan, *LC•GC Eur.*, **18**(4), 196–203 (2005).
2. U.D. Neue et al., *J. Chromatogr. A*, **849**, 87 (1999).
3. M. Kele and G. Guiochon, *J. Chromatogr. A*, **869**, 181 (2000).
4. J.W. Dolan et al., *J. Chromatogr. A*, **960**, 51 (2002).
5. L.R. Snyder, J.J. Kirkland and J.L. Glajch, *Practical HPLC Method Development*, 2nd ed. (WileyInterscience, New York, USA, 1997).
6. "Guidance for Industry: Bioanalytical Method Validation," FDA (May 2001).
7. "Guidance for Industry: Q2B Validation of Analytical Procedures: Methodology," ICH (November 1996).
8. The United States Pharmacopoeia, *USP 28*, Rockville, Maryland, USA (2005).
9. R. Cox and G. Meillon, *PharmEuropa*, **10**, 58 (1998).
10. W.B. Furman, J.G. Dorsey and L.R. Snyder, *Pharm. Technol.*, **58** (June 1998).
11. *Pharmacopoeial Forum*, **30**(3), 1015–1017 (May/June 2004).
12. "Guidance for Industry: Analytical Procedures

- and Methods Validation," FDA (August 2000).
13. "Guideline for Industry: Text on Validation of Analytical Procedures," ICH-Q2A (March 1995).
14. J.W. Dolan, D.C. Lommen and L.R. Snyder, *J. Chromatogr.*, **535**, 55 (1990).

Lloyd R. Snyder is with LC Resources, 26 Silverwood Ct., Orinda, California, USA. He can be reached at snyder0036@comcast.net.

John W. Dolan is with BASi Northwest Laboratory, McMinnville, Oregon, USA. He can be reached at John.Dolan@Bioanalytical.com.

"Column Watch" editor **Ronald E. Majors** is Business Development Manager, Consumables and Accessories Business Unit, Agilent Technologies, Wilmington, Delaware, USA and is a member of the Editorial Advisory Board of *LC•GC Europe*. Direct correspondence about this column to *LC•GC Europe*, Advanstar House, Park West, Sealand Road, Chester CH1 4RN, UK, e-mail: dhills@advanstar.com