Variability of column selectivity for reversed-phase high-performance liquid chromatography Compensation by adjustment of separation conditions

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Abstract

Reversed-phase columns are widely used in assays based on high-performance liquid chromatography (HPLC). When such assays are repeated over time, it is often necessary to replace the column. In such cases, the selectivity of columns from different production batches may prove sufficiently variable to result in a failed separation. It is possible to compensate for differences in column selectivity by making small changes (adjustments) in separation conditions. The present paper describes an efficient procedure for choosing adjusted conditions and discusses its general applicability. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Countless reversed-phase HPLC assays are carried out every day. Many of these procedures are applied over a period of months or years, during which time it is usually necessary to replace the column one or more times (due to the deterioration of column performance with use). The replacement column is normally one with the same part number, which it is expected will provide an equivalent separation of the sample. However, the selectivity of different production batches of nominally similar column packing is never exactly the same; for some combinations of column packing, sample and separation conditions, it will be found that sample retention and resolution can vary unacceptably from batch to batch. Surveys

of column users over the period 1991–1997 [1,2] report that column-to-column reproducibility is their single most important consideration when selecting a column supplier, and instances of unacceptable column variability have been reported in the literature ([3,4a] and review of [4b]). Since 1995, several additional (unpublished) examples of column variability have come to our attention.

More recently, a series of ongoing studies has been published [5–7] concerning the variability of columns produced by major manufacturers. For columns produced at the present time, these investigations suggest that column reproducibility is no longer a major problem. This conclusion is supported by a separate study [8] of columns produced by one of the largest suppliers of RP-LC columns, where two conclusions were drawn from a very large data base. First, it was found for any one column type (e.g. μBondapak) that column reproducibility has improved over time—presumably as a result of better

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controlled manufacturing processes. Second, for different kinds of columns, it was found for the period 1991–1998 that columns of newer design (e.g. Symmetry C_{18}) are more reproducible than are older columns such as Novapak or μBondapak. Together, the data of Refs. [5–8] suggest that column variability may be of less concern today than in the past.

There are reasons, however, for suspecting that column variability will be a continuing (if decreasing) problem. First, the test procedures employed for the purpose of establishing column reproducibility in the studies of Refs. [5–8] and elsewhere are usually restricted to a small number of sample probes and separation conditions. The use of these and other column test procedures cannot guarantee that two column batches with identical test results will provide identical retention for other samples and/or separation conditions. Studies under way in our laboratory [9] suggest that improved column test procedures are possible, with the ultimate goal of being able (with a small number of general tests) to ensure that different column batches will provide similar separations for most samples and separation conditions.

Second, many RP-LC methods carried out today were developed several years ago and continue to use columns of earlier design. Column variability is likely to remain a potential problem for at least some of these older assay procedures. Finally, column variability can be a problem for a single column as a result of changes in retention and selectivity during use (column aging).

There are several possible ways in which the problem of column variability can be addressed:

1. Replace the ''problem'' column with one from another batch, or even one of ''similar'' kind (but different part number or manufacturer). This approach is often attempted when the problem of column variability first arises, but the resulting trial-and-error search for a suitable column replacement can be inefficient and (in our experience) is often unsuccessful.
2. Re-optimize separation conditions to obtain an acceptable separation for the ''problem'' column (and presumably other columns from more recent batches) or a different column. This procedure is likely to prove successful, but it is time consum-

ing, especially when the need for a complete method re-validation is considered.
3. During method development, select conditions that will provide acceptable separation on more than one kind of column [10]. This provides one or more alternative columns, for use if the problem of column variability should arise for any one of these columns.
4. Adjust (vary) separation conditions for the new column so as to restore the original separation. Guidelines for maximum values of these adjusted conditions have been reported [11,12] for use with regulated assay procedures. If successful, method adjustment in this way should require only minimal revalidation.

The present paper examines the last option (method adjustment) in more detail, and presents some general procedures for making this procedure more effective and efficient.

2. Theory and approach

In the present study, we have simulated column variability using two column batches, one a commercial C_{18} column (SB-100), and the other a column (SB-90) with a stationary phase made from identical starting materials, but with the silanization reaction stopped at 90% bonding (see Experimental). Percent-bonding typically varies slightly for commercial columns from batch-to-batch, but usually by less than this 10%. The latter two columns provide a possibly exaggerated example of column variability, one which should therefore pose a suitable challenge to method adjustment as a correction for column differences. As samples, we have used random combinations of compounds from the 67 solutes shown in Table 1. Both isocratic and gradient separations were carried out using acetonitrile/buffer mobile phases, with conditions varied so as to determine changes in separation as a function of four variables: temperature, T; mobile phase strength (%B [isocratic]), or gradient time t_G; mobile phase pH; buffer concentration. For changes in T and either %B or t_G, it was possible to use our experimental data to predict separation as a function of simultaneous changes in both variables via computer simulation [13].

Table 1
Compounds used in the present study

A. Neutral solutes			B. Basic solutes	C. Acidic solutes (weak acids)
1. Benzene	16. *N*-Benzyl-formamide	31. Acetophenone	*B.1. Strong bases*	56. Diclofenate acid
2. Toluene	17. Anisole	32. Benzophenone	46. Amitriptyline	57. Mefenamic acid
3. Ethylbenzene	18. Benzyl alcohol	33. *cis*-Chalcone	47. Diphenhydramine	58. Ketoprofen
4. *p*-Xylene	19. 3-Phenyl propanol	34. *trans*-Chalcone	48. d,1-Propanolol	59. Diflunisal
5. Propylbenzene	20. 5-Phenyl pentanol	35. *cis*-4-Nitro-chalcone	49. Nortriptyline	60. 4-*n*-Butylbenzoic acid
6. Butylbenzene	21. Phenol	36. *trans*-4-Nitro-chalcone	50. Prolintane	61. 4-*n*-Pentylbenzoic acid
7. Napthalene	22. *p*-Chlorophenol	37. *cis*-4-Methoxy-chalcone	*B.2. Weak bases*	62. 4-*n*-Hexylbenzoic acid
8. *p*-Chlorotoluene	23. 2,3 Dihydroxy-napthalene	38. *trans*-4-Methoxy-chalcone	51. 4-Pentyl aniline	63. 3 Cyanobenzoic acid
9. Dichlorobenzene	24. 1,3 Dihydroxy-napthalene	39. Prednisone	52. 4-Hexyl aniline	64. 2-Nitrobenzoic acid
10. Benzotrichloride	25. Eugenol	40. Hydrocortisone	53. 4-Heptyl aniline	65. 3-Nitrobenzoic acid
11. Bromobenzene	26. Danthron	41. Mephenytoin	54. *N*-Ethylaniline	66. 2,6-Dimethylbenzoic acid
12. 1-Nitropropane	27. *n*-Propyl formate	42. Oxazepam	55. 2-Phenyl pyridine	67. 2-Fluorobenzoic acid
13. Nitrobenzene	28. Methylbenzoate	43. Flunitrazepam		
14. *p*-Nitrotoluene	29. Benzonitrile	44. 5,5 Diphenyl-hydantoin		
15. *p*-Nitrobenzyl chloride	30. Coumarin	45. *N,N*-Dimethyl acetamide		

2.1. An example of column variability and its correction by method adjustment

Fig. 1 uses a five-component sample to illustrate two approaches to method adjustment. In Fig. 1a, the initial separation on the "good" column (SB-100) gives a "critical" resolution $R_s^* = 1.9$. Replacement of the latter column by the "bad" column (SB-90) in Fig. 1b, shows a serious loss in resolution: $R_s^* = 1.1$. (note: in the present paper, R_s refers to the resolution of any band-pair, while R_s^* refers to the critical resolution of the entire chromatogram; i.e. the value of R_s for the poorest resolved band-pair).

2.1.1. Empirical method adjustment

One approach to method adjustment is to vary conditions by trial-and-error, in an effort to restore the original resolution. Such a result is illustrated in Fig. 1c, where an increase in %B to 54.5% results in $R_s^* = 1.8$. While the latter result (c) has essentially restored the "critical" resolution found in (a) for the "good" column, it can be seen that these two separations are somewhat dissimilar. This is better shown in Fig. 2a,c. In (a), retention times from Fig. 1c are plotted versus retention times from Fig. 1a. Retention times for the two runs differ by an average factor of 0.79, which could be corrected by a change in flow-rate for Fig. 1c by a factor of (1/0.79). However, even with this flow-rate adjustment, re-

tention times would still differ significantly, as indicated by the standard error of the correlation of Fig. 2a (±0.28 min). An even greater discrepancy between the runs of Fig. 1a,c is shown by comparing values of R_s for all adjacent band-pairs for the two columns (Fig. 2c), which shows essentially no correlation of resolution values.

While method adjustment as in Fig. 1c might meet the needs of some assay procedures, it is generally preferable if the separation on the "bad" column (with adjusted conditions) matches that for the "good" column as closely as possible. Thus, the method procedure may specify retention time windows for each peak, which requires that retention times for each analyte fall within a narrow range. Significant changes in t_R for a given peak can also result in changes in detection sensitivity, which in turn may affect the precision of quantitation. Finally, whenever retention is markedly different for the adjusted separation and "bad" column, it can be argued that method performance as established by validation of the original method (using a "good" column) should not be assumed to remain exactly the same.

2.1.2. An alternative method adjustment procedure

A preliminary version of an efficient, general procedure has been described [10] for adjusting conditions so as to match separations on "good" and
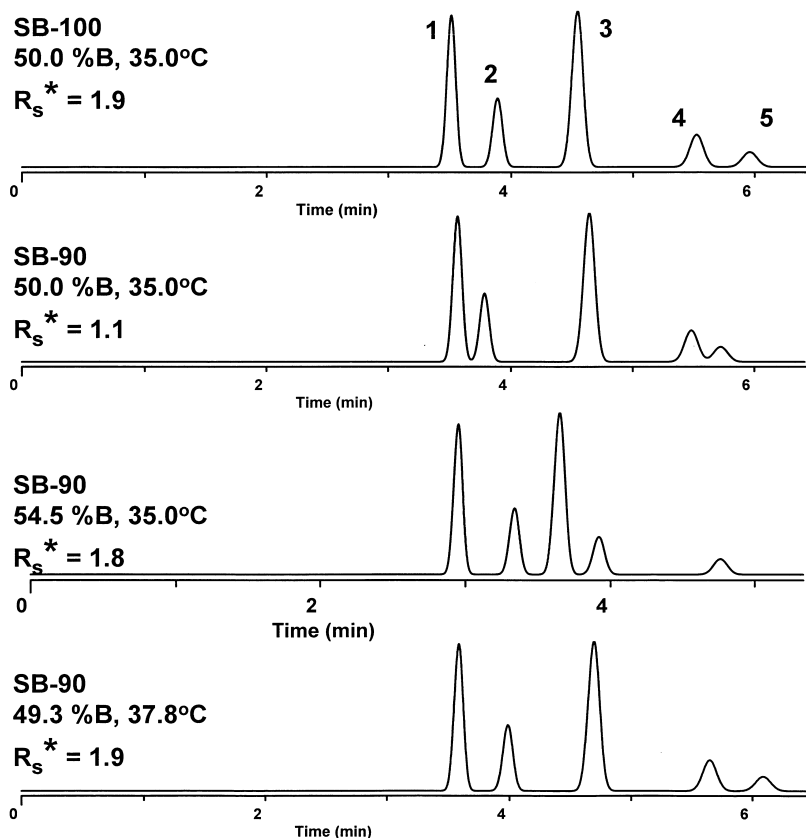
Fig. 1. Example of method adjustment. Sample composed of compounds from Table 1; #1=28, #2=51, #3=14, #4=52, #5=60. (a) SB-100 column with isocratic conditions as shown; (b) SB-90 column, same conditions as in (a); (c) SB-90 column, arbitrary adjustment of conditions to restore resolution of (a); (d) SB-90 column, conditions adjusted via Eq. (1). DryLab 2000 simulations.

''bad'' columns. The application of this procedure to the example of Fig. 1a,b results in the separation of Fig. 1d. The comparison of values of t_R and R_s for this adjusted run (d) versus values for the ''good'' column (a) is shown in Fig. 2b,d. It is seen for values of both t_R and R_s that a very close agreement now exists for the adjusted ''bad'' versus ''good'' columns. The application of this method adjustment procedure assumes that experimental runs have been carried out (for either column) in which one or more conditions are individually changed by a small amount. For the example of Fig. 1, where T and %B were each varied during method adjustment, the required ''off-set'' runs for the ''good'' column are shown in Fig. 3b,c, and compared in (a) with the original separation of Fig. 1a. Method validation usually includes the determination of ''off-set'' runs

as in Fig. 3, in order to establish method robustness or to help diagnose mis-calibrated equipment or mistakes in separation conditions [14]. Off-set runs will therefore often be available when method adjustment is required. When this is not the case, off-set runs can be carried out on the ''bad'' column.

Returning to the example of Figs. 1 and 3, method adjustment begins with the calculation of values of R_s for each adjacent band-pair in: (a) the separations on ''good'' and ''bad'' columns (Fig. 1a,b) and (b) the off-set runs of Fig. 3b,c. Changes in R_s (δR_s) versus the ''good'' separation of Fig. 1a are next calculated for corresponding band-pairs. The latter values of δR_s are then divided by R_s for the ''good'' separation, yielding values of $\delta R_s / R_s$ for the latter three runs (Figs. 1b, 3b,c). Finally, values of $\delta R_s / R_s$ for the off-set runs are divided by the change in the
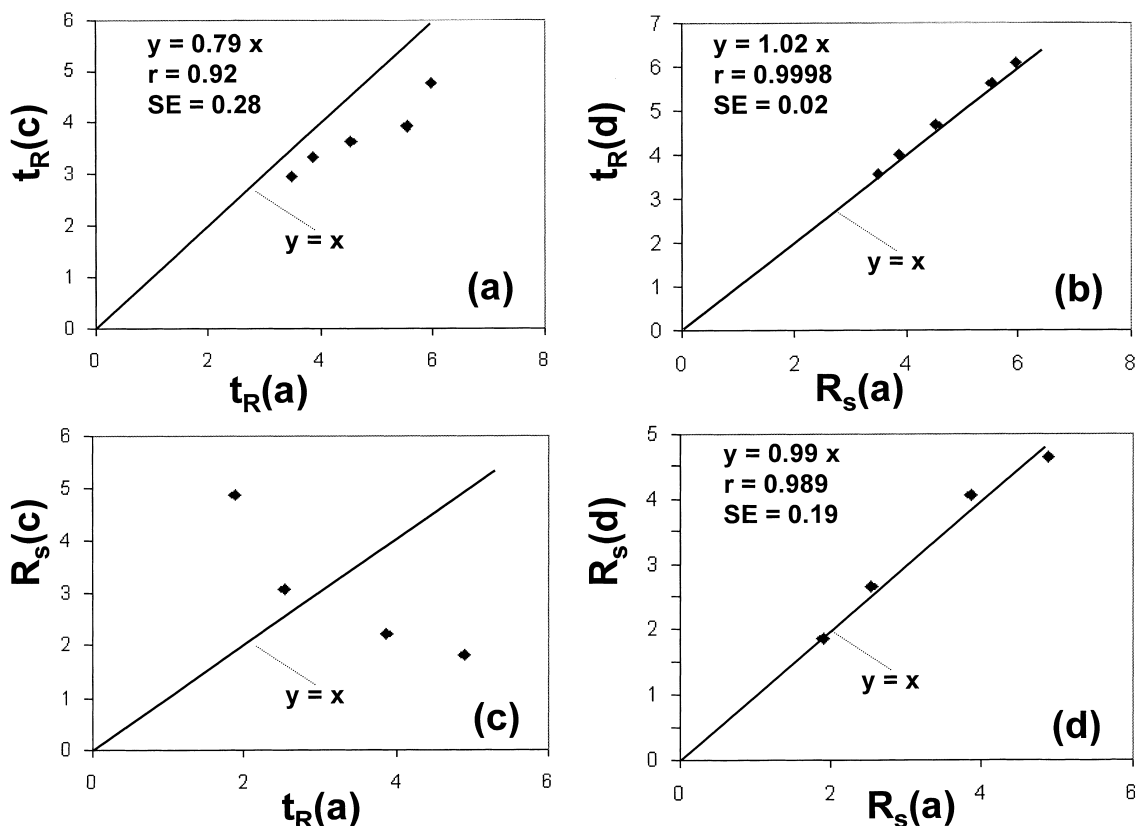
Fig. 2. Comparison of retention time t_R and resolution R_s for adjacent bands in adjusted separations of Fig. 1 versus values in Fig. 1a for SB-100 column. (a, c) Values for separation of Fig. 1c versus 1a; (b, d) values for separation of Fig. 1d versus 1a.

corresponding variable X (ΔX), to give relative changes in resolution per unit change in the condition: $(\delta R_s/R_s)/\Delta X$. If values of $\delta R_s/R_s$ for the "bad" column (for different adjacent band-pairs) are compared with corresponding values of $(\delta R_s/R_s)/\Delta X$ for the off-set runs via multiple regression, there results a minimum-least-squares-difference solution of the form

$$- \delta R_s/R_s \text{ ("bad column")} = x_1 (\delta R_s/R_s)/\Delta X_1$$
$$+ x_2 (\delta R_s/R_s)/\Delta X_2 + \cdots \qquad (1)$$

The coefficients x_1, x_2, ... now correspond to the necessary change in each of the conditions X_1, X_2, ... for a method adjustment that will minimize differences in $\delta R_s/R_s$ for the "bad" versus "good" columns (least-squares fit). The latter procedure results in the adjusted conditions and separation of

Fig. 1d. A more detailed description of this method adjustment procedure is provided by the example of Appendix A.

The use of $\delta R_s/R_s$ in the regression of Eq. (1) (as opposed to values of R_s) recognizes that changes in R_s for "bad" versus "good" columns are usually more important for "critical" band-pairs (those with the smallest values of R_s, equal to R_s^*). In those cases where a loss of separation is understated by a value of R_s (e.g. small bands adjacent to large bands), it can be advantageous to provide a greater weight to these bands in the regression. This is readily accomplished (by trial and error) using standard spreadsheet programs that support multiple regression analysis.

Selection of adjusted conditions via Eq. (1) is based solely on the closest possible match of relative R_s values, rather than retention times. Thus, a good
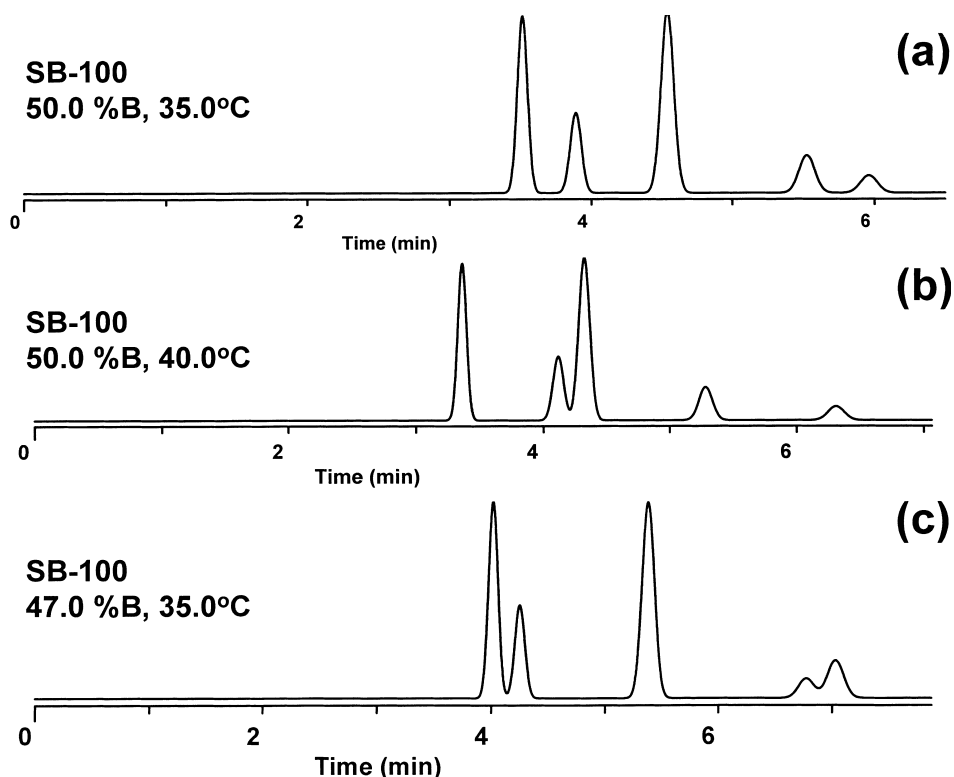
Fig. 3. Off-set runs for separation of Fig. 1a. (a) Same separation as in Fig. 1a; (b) same as in (a), except for indicated change in temperature *T*; (c) same as in (a), except for indicated change in %B. DryLab 2000 simulations.

match for R_s may be accompanied by a proportional change in all retention times to higher or lower values. In such cases, isocratic retention can be adjusted further by a change in flow-rate, with little effect on resolution for separations that involve efficient, small-particle columns. When flow-rate is changed for the same purpose in gradient separations, it is important to change the adjusted gradient time t_G in inverse proportion to the change in flow-rate, in order to maintain the same band spacing or selectivity [15].

It can be more convenient to select off-set runs for the ''good'' column, as these data are usually available as part of method validation. However, off-set runs for the ''bad'' column can also be used, if values of δR_s for these runs are calculated as the difference in R_s values for each off-set run versus the ''bad'' column. The use of off-set runs for the ''good'' column to determine changes in conditions

for the ''bad'' column assumes that the effect of a change in conditions on values of log k is identical for both columns. We have shown that this is generally so [9], especially for similar columns (as would be the case for different batches of nominally equivalent columns). It is further assumed that the effect on retention and resolution of changing several conditions simultaneously can be predicted from runs where conditions are changed one-at-a-time. This approximation is less likely to be reliable for relatively large changes in each condition, but we have shown previously that the latter is a good approximation for moderate changes in each condition [16]. Error in predicted retention times can arise as a result of larger changes in conditions, in which case the recommended changes in conditions from Eq. (1) can be slightly in error. Such errors can be corrected simply by repeating the application of Eq. (1), in this case replacing the R_s values from the original

separation on the ''bad'' column with values from the separation on the ''bad'' column for conditions initially recommended by Eq. (1).

2.1.3. Choice of adjustable conditions

In some cases, a satisfactory method adjustment can be achieved by varying column temperature and either isocratic %B or gradient time. Often a more complete adjustment results, when additional conditions are used for adjustment (see Results and discussion). Only those conditions which can affect band spacing (selectivity) are useful in this regard, e.g. changes in flow-rate or column dimensions will *not* affect isocratic band spacing or the relative retention of different band pairs, nor will change in pH or buffer concentration significantly affect the retention of compounds other than acids or bases. In the case of gradient separations, changes in flow-rate F, column dimensions or gradient time t_G each result in equivalent changes in peak spacing; change in t_G is usually preferred to change in either F or column dimensions.

In the present study, we have varied temperature and mobile phase composition (%B, pH and buffer concentration). For more complex mobile phases used with the ''good'' column, additional conditions may be available for adjustment: concentration of an ion-pair reagent or other mobile phase additive, concentrations of a second organic solvent (methanol, acetonitrile, tetrahydrofuran), etc. However, we do not recommend the addition to the mobile phase of components not originally present, e.g. ion-pair reagents, new organic solvents, etc. Allowable changes or adjustment in various conditions have been suggested in Refs. [11,12].

3. Experimental

3.1. Experimental procedures and materials

Results presented here are based on experimental data reported in Refs. [9]. The ''good'' column is an Agilent Stablebond C_{18} column (Agilent, Newport, DE) from a production batch, identified here as ''SB-100''. Using identical silica and silane reagents, the manufacturer produced a second (research) batch of stationary phase by adjusting reaction conditions

so as to provide a reduced silane coverage of 1.79 μmol/m^2 (vs. 2.08 μmol/m^2 for the production column). Columns packed with the under-bonded material are identified as ''SB-90'' and are referred to as ''bad'' columns. Experimental conditions for unadjusted isocratic runs used 15×0.46-cm columns, a flow-rate of 1.5 ml/min., and mobile phases of acetonitrile/phosphate (31.5 mM, pH 2.8). For gradient experiments, the mobile phase was 5–80% acetonitrile/phosphate (10 mM, pH 2.8).

3.2. Simulation software

Based on the experimental data of Ref. [9], it was possible to carry out simulations of separation as a function of T and either %B or t_G, using DryLab 2000 software (LC Resources). For convenience, a representative value of $N = 10\,000$ was assumed for the latter simulations (any difference in actual N values would result in proportional changes in all R_s values, with no effect on the present conclusions). Method adjustments based on Eq. (1) were carried out using a home-made program (LC-Fixit) based on an Excel spreadsheet (Microsoft Windows). LC-Fixit allows the entry of either band-width or resolution values as a means of calculating δR_s values and the coefficients of Eq. (1), thereby determining final adjusted conditions. The latter program also permits empirical, trial-and-error adjustments of conditions for improved separation, as well as the use of weighting factors (with Eq. (1)) for individual band-pairs. Adjustments of retention apart from resolution via changes in flow-rate are also predicted by LC-Fixit.

3.3. Simulation procedures

Two sets of experimental data from Ref. [9] were used in this study: (a) isocratic measurements for the SB-100 and SB-90 columns with a mobile phase of 50% acetonitrile/phosphate buffer (31.5 mM, pH 2.80) and $T = 35\,°C$), plus off-set runs for change in individual conditions: T (45 °C), %B (40%B), pH (2.7 and 2.9), and buffer concentration (16.7 mM); (b) gradient measurements for $T = 35$ and 50 °C, and $t_G = 10$ and 20 min, suitable for use with DryLab 2000 to predict separation as a function of T and t_G. Data from (a) allowed the application of Eq. (1) with

T, %B, pH and buffer concentration as variables X_1, X_2, etc. Samples were chosen as random combinations of the compounds of Table 1. Data from (b) were used to select either isocratic or gradient conditions for random samples from Table 1 and the two columns (SB-100, SB-90), so as to provide acceptable resolution for each sample with the SB-100 ("good") column ($R_s^* > 1.5$) and unacceptable resolution ($R_s^* < 1.5$) for the SB-90 ("bad") column (using the same conditions for each column). DryLab 2000 simulations were used with each sample for (a) the selection of T and t_G values which fulfil the latter requirement, (b) the similar prediction of off-set runs where T and t_G were varied, and (c) the prediction of final separations with adjusted conditions. For the range in T (15 °C) and t_G (factor of two) used in the present study, computer simulation provides predictions which are as reliable as actual experiments [17].

4. Results and discussion

4.1. Application of Eq. (1) to samples selected from the compounds of Table 1

4.1.1. Isocratic simulations based on isocratic experiments

It was anticipated that method adjustment will prove more difficult, as the number n of sample components increases. In order to evaluate the likely success of method adjustment as a function of n, a series of random samples (with n varying) were selected from the list of Table 1, and method adjustment was carried out as in Fig. 1d by means of the procedure described in Appendix A (based on Eq. (1)). The evaluation of how well method adjustment can compensate for differences in two separations (SB-100 vs. SB-90 columns) was made in terms of the coefficient of variation (CV) of the quantity $\delta R_s / R_s$ for each band-pair in the two separations. Here, δR_s refers to the difference in values of R_s for the adjusted "bad" column (SB-90) versus the unadjusted "good" column (SB-100). The value of R_s in the quantity $\delta R_s / R_s$ refers to the "good" column. Thus, $\delta R_s / R_s$ measures the fractional difference in R_s for a given band-pair between "good" and adjusted "bad" columns. In the exam-

ple of Fig. 1d, the CV for values of ($\delta R_s / R_s$) is 5%, versus a value of 35% for the unadjusted "bad" column (Fig. 1b). Assuming that the initial separation on the "good" column has $R_s^* \geq 2.0$ (a typical target for method development), and baseline resolution ($R_s^* \geq 1.5$) is required for the adjusted "bad" column, we require a value of CV$\leq$25% for values of $\delta R_s / R_s$. A more conservative requirement for successful method adjustment is assumed in the following discussion: CV$\leq$15%.

Fig. 4 summarizes values of CV($\delta R_s / R_s$) for a large number of samples (6–10 samples for each value of n). The initial conditions for the "good" column were held constant: 50% acetonitrile/buffer, 35 °C, 31 mM buffer, pH 2.80. In this case, offset runs were carried out for changes in T and %B. As expected, values of CV($\delta R_s / R_s$) are smaller for the adjusted (vs. unadjusted) separations, with CV($\delta R_s / R_s$) for the unadjusted runs averaging three times greater (Fig. 4). Values of CV($\delta R_s / R_s$) also increase with n, as expected. For a maximum CV($\delta R_s / R_s$) value of 15%, $n \leq 14$; i.e. method adjustment based on Eq. (1) with a change in T and %B should be successful in a majority of cases, as long as the sample contains no more than 14 components. However, this conclusion assumes samples similar to
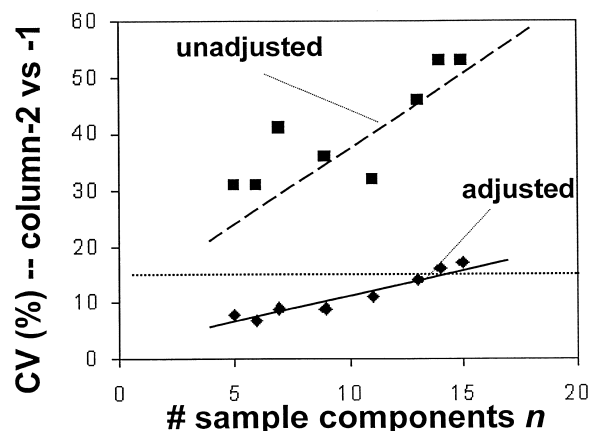

Fig. 4. Efficacy of method adjustment (Eq. (1)) for about 80 randomly selected samples composed of the compounds of Table 1. Coefficient of variation (CV) values for differences in resolution between "good" and "bad" columns (SB-100 and SB-90, respectively). Data points for each value of n are an average of 6–10 samples each.

those used in the data of Fig. 4 (see the later discussion in Section 4.2).

Method adjustment (varying T and %B or t_G may prove successful for more complex samples (larger n), but the probability of success is then <50%.

The application of Eq. (1) to those samples of Fig. 4 which contained one or more ionizable compounds was repeated for other combinations of adjustable conditions, in order to determine the dependence of $CV(\delta R_s/R_s)$ on the choice of conditions used for method adjustment. Relative to the case where only T and %B were adjusted, $CV(\delta R_s/R_s)$ was 0.83 as large when T, %B and pH were adjusted simultaneously. Similarly, $CV(\delta R_s/R_s)$ was 0.73 as large when T, %B, pH and buffer concentration were adjusted. In other words, adding pH and buffer concentration (for a total of four variables) significantly improves the ability of Eq. (1) to correct for column differences, when ionizable compounds are present in the sample. This suggests, for samples containing ionizable compounds, that method adjustment should prove successful in the majority of cases where $n \leq 18$, when T, %B, pH and buffer concentration are simultaneously varied for method adjustment.

4.1.2. Simulations based on gradient measurements

The following study was based on Drylab 2000 simulations, using as input experimental gradient separations where both T and t_G had been varied. Predictions of either isocratic or gradient separation were then possible, as a function of simultaneous changes in T and either %B or t_G. Similar simulations were used in Ref. [10] to show the ability of Eq. (1) to adjust conditions for the minimization of differences in resolution among different columns for a single sample. Unlike the experiments of the preceding section and Fig. 4 based on fixed conditions for the "good" column (50%B, 35 °C), conditions of T and either %B or t_G for the following separations on the "good" column were chosen so that critical resolution $R_s^* \geq 1.5$, corresponding to more realistic examples of separation. At the same time, these same conditions of T and %B were also required to yield a distinctly lower resolution for the "bad" column—thereby making method adjustment necessary. This process is illustrated in Fig. 5, which shows representative maps for a different sample and

the present two columns (SB-100 and SB-90). It is possible to select the same conditions T and t_G for the two columns so as to provide similar and acceptable resolution (e.g. 25.5%B and 43 °C; $R_s^* = 1.7–1.8$). However, such a choice of conditions would not require method adjustment for this sample. Therefore, identical conditions were selected for each of these experiments (24%B, 45 °C; cross-hairs of Fig. 5) such that the resolution for the "bad" column was substantially lower than for the "good" column: $R_s^* = 1.9$ for the "good" column (Fig. 5a) versus 1.0 for the "bad" column (Fig. 5b). Method adjustments based on simulations of this kind were intended to allow us to: (a) confirm some of the conclusions of Fig. 4 and (b) examine more closely the reasons for any failures of method adjustment, especially where method adjustment provides unacceptable resolution.

Isocratic experiments for a total of 12 samples with $n = 10$ (10 components in each sample) were examined as described above. A typical example of a successful method adjustment is illustrated in Fig. 6 for sample #11 of the present set. The separation on the "good" column (a) has $R_s^* = 1.8$, while $R_s^* = 1.0$ (arrow) on the "bad" column (b). Method adjustment (c) results in $R_s^* = 1.8$, identical to that in (a); the CV for $(\delta R_s/R_s)$ values in (c) is 16%. Retention times in (c) are on average 8% greater than in (a); this difference in separation can be minimized by increasing flow-rate by 8% (d) to a value of 1.62 ml/min (vs. 1.50 ml/min in [a]). The vertical dotted lines in Fig. 6 illustrate the success of this final adjustment in minimizing differences in t_R between (a) and (d). However, an exact match of retention times by method adjustment plus flow-rate changes is seldom possible.

Similar gradient simulations for seven samples with $n = 10$ were also carried out. Fig. 7 illustrates the results for one of these samples. In this case, method adjustment (c) results in a value of $R_s^* = 1.8$ for the "bad" column, versus an unadjusted value for the "bad" column of $R_s^* = 1.2$ (b), and a resolution for the "good" column (a) of $R_s^* = 2.1$.

For the 21 samples separated by isocratic or gradient elution, average resolution values for the "good" column, "bad" column and adjusted "bad" column, were $R_s^* = 1.9$, 0.8 and 1.6, respectively. That is, method adjustment according to Eq. (1)
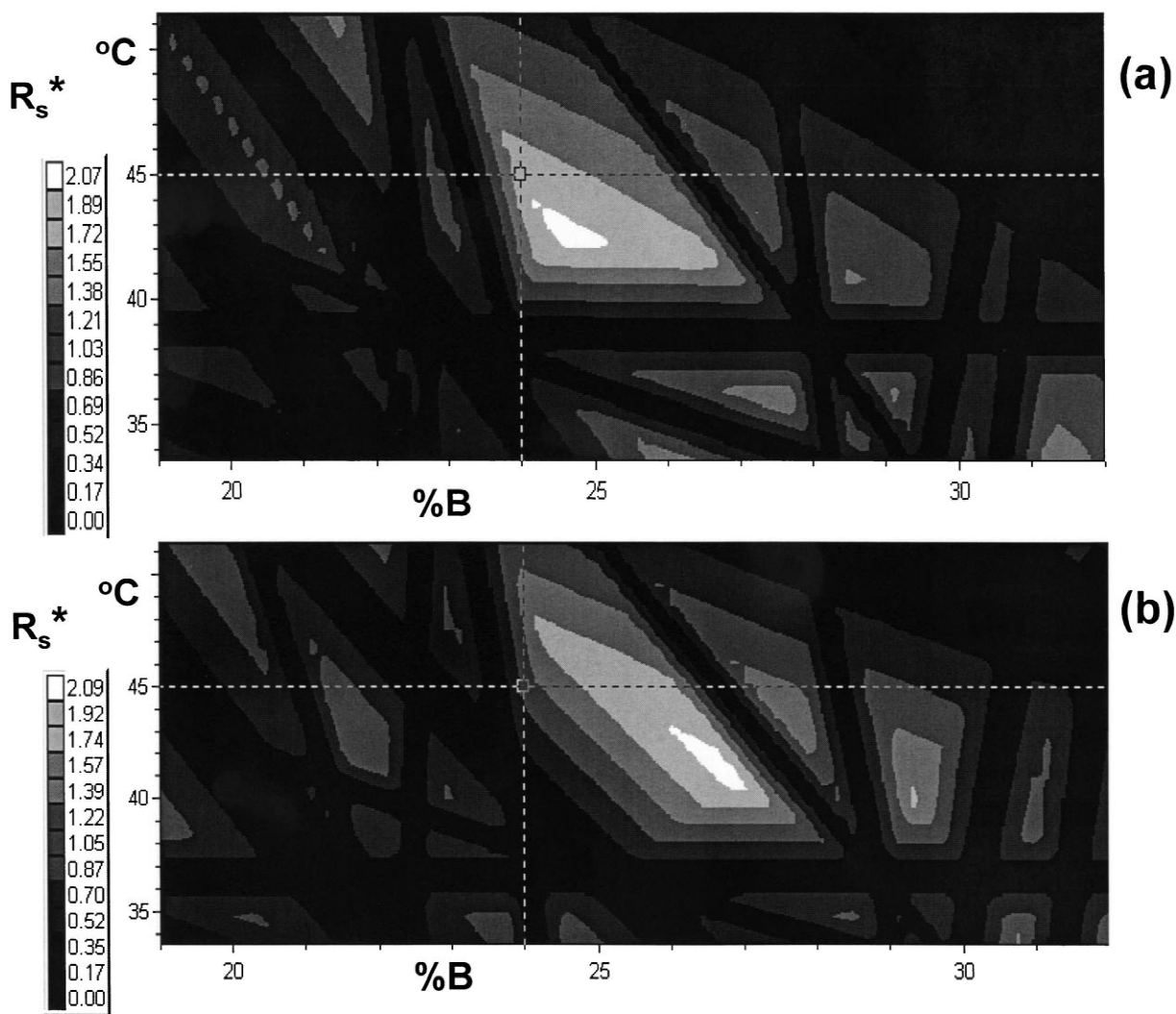
Fig. 5. Resolution maps (plots of R_s^* vs. T and %B) for a 10-component sample (#10, 18, 19, 21, 23, 27, 48, 55, 63, 66) as a function of temperature and %B; (a) ''good'' column (SB-100); (b) ''bad'' column (SB-90).

largely compensated for differences in critical resolution for ''good'' versus ''bad'' columns. Based on our previous discussion, an acceptable adjustment should result in a critical resolution that is at least 85% as large as that on the ''good'' column. Of the present 21 samples, 2/3 of the adjusted separations met this criterion.

For the remaining one-third (seven) samples, method adjustment via Eq. (1) was less successful. Corresponding average values of R_s^* were 1.9 (''good'' column), 0.7 (''bad'' column), and 1.3

(''bad'' column after adjustment). That is, method adjustment via Eq. (1) corrected for some, but not all, of the differences in critical resolution between ''good'' and ''bad'' columns. Further adjustment of conditions was therefore pursued, based on either (a) giving a higher weight in the regression of Eq. (1) for certain critical band-pairs or (b) trial-and-error variation of T and %B around the adjusted values recommended by Eq. (1) (either option is supported by LC-Fixit). These further adjustments led to an acceptable separation for four out of the remaining
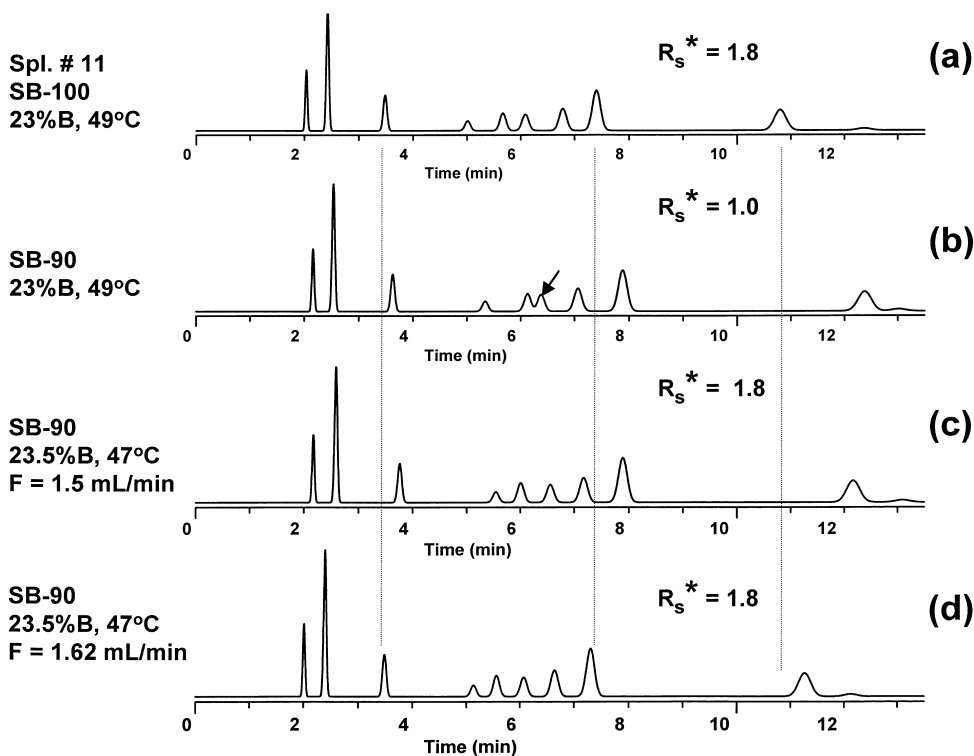
Fig. 6. Successful method adjustment for an isocratic method, based on Eq. (1) and changes in temperature and solvent strength (%B). Sample composed of compounds #18, 19, 23, 24, 31, 40, 44, 48, 65, 67 of Table 1. (a) Separation on ''good'' column (SB-100); (b) separation on ''bad'' column (SB-90); (c) adjusted separation based on Eq. (1); (d) further adjustment of flow-rate to minimize differences in retention time versus (a). Other conditions noted in figure. DryLab 2000 simulations.

seven samples, so that the adjustment of 18 out of 21 samples was eventually successful (via Eq. (1) or empirically). An example of trial-and-error adjustment is provided in Fig. 8. The separation on the ''good'' column (a) has $R_s^* = 1.8$, versus 0.1 for the ''bad'' column (b). Method adjustment based on Eq. (1) yields the partially corrected separation of (c), with $R_s^* = 1.5$. Further trial-and-error changes in conditions provide the final separation of (d), with $R_s^* = 1.8$ (same as for the separation of [a]). Retention times in (d) are on-average smaller than in (a), but this difference can be minimized by a change in flow-rate, as in Fig. 6d.

We note also for the preceding 21 samples that method adjustment was similarly successful as for the samples of Fig. 4 (as measured by the CV of $[\delta R_s / R_s]$ values). Thus, for $n = 10$, Fig. 4 predicts a CV($\delta R_s / R_s$) value of 11%. For the samples of the present section ($n = 10$, as in Figs. 6–8), the average

CV($\delta R_s / R_s$) was 14%. Similarly, for a smaller number of samples where $n = 5$, values of CV($\delta R_s / R_s$) were 7% for both cases (Fig. 4 and simulations based on gradient experiments).

4.2. Reasons for a failure of Eq. (1) to provide full compensation for column variability

There are various reasons for a failure of method adjustment to adequately compensate for differences in column selectivity and fully restore critical resolution:
- a critical band-pair whose resolution is little affected by different changes in conditions;
- presence of two or more critical band-pairs, whose respective values of R_s cannot be independently varied by adjusting conditions;
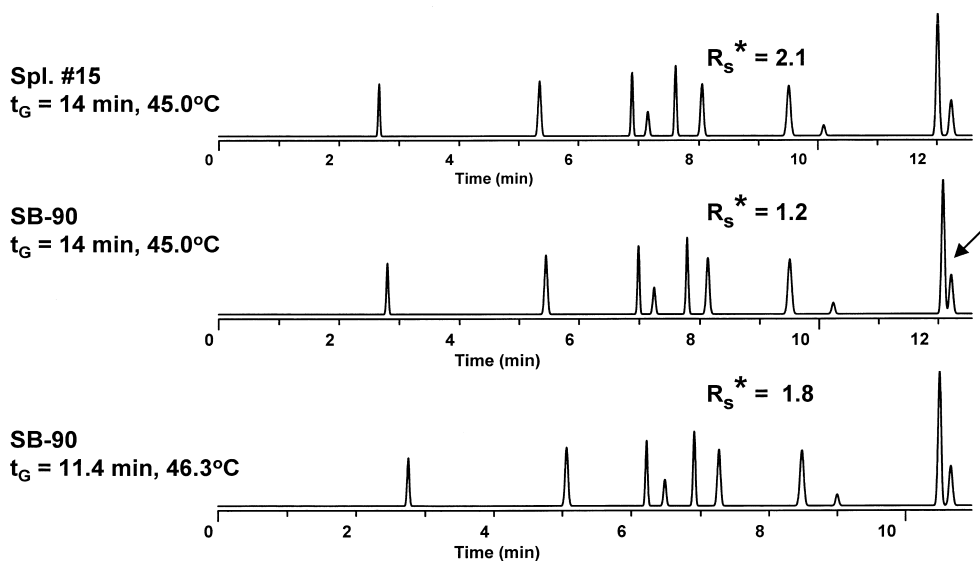- impractical changes in conditions suggested by the use of Eq. (1).

Fig. 7. Successful method adjustment for a gradient method, based on Eq. (1) and changes in temperature and solvent strength (%B). Sample composed of compounds #1, 9, 18, 20, 22, 24, 37, 39, 45, 48 of Table 1. (a) Separation on ''good'' column (SB-100); (b) separation on ''bad'' column (SB-90); (c) adjusted separation based on Eq. (1). Other conditions noted in figure. DryLab 2000 simulations.

Each of these three possibilities are examined next.

4.2.1. Band-pairs unseparated by changes in T and %B

The preceding study of 21 samples as a test of method adjustment did not reveal any examples of critical band-pairs whose resolution was insufficiently responsive to changes in T and/or %B. A total of 258 adjacent or near-adjacent band-pairs from the compounds of Table 1 were studied as a function of simultaneous changes in T (0–15 °C) and %B (0–5%B). In only 6% of these cases (15 band pairs) was there a maximum potential change in resolution less than 1.5 R_s-units for these changes in T and %B, suggesting that a modest adjustment of T and %B might not have been able to achieve acceptable resolution ($R_s \geq 1.5$) for some of these band-pairs. However, the compounds of Table 1 are quite diverse in terms of structure, whereas samples composed of structurally related compounds would likely show a higher percentage of band-pairs whose resolution is insufficiently sensitive to changes in T and %B [18]. Isomeric band pairs constitute an extreme example of structural similarity, and a recent study has reported the separation of such isomers as

a function of changes in T and %B [19]. The latter study suggests that only 10% of all isomers will show changes in resolution of <1.0 R_s-units for practical changes in T and %B. On balance, we believe that method adjustment based only on changes in T and %B will fail for less than 10% of all isocratic separations, as a result of critical band-pair resolution that is insufficiently affected by change in these conditions. A lower failure rate can be expected for: (a) gradient elution (because of a wider range of acceptable retention factors $k*$ [15]) or (b) method adjustment that involves change in additional conditions (e.g. pH, buffer concentration).

4.2.2. Two or more critical band pairs

Method adjustment seldom allows a fully independent control of resolution for all band pairs. There are only a limited number of conditions available for the control of resolution, while the number of adjacent bands-pairs requiring control usually exceeds the number of conditions to be adjusted. When two or more potentially critical band-pairs are present in a chromatogram, the latter problem is further aggravated, as can be seen in the example of Figs. 9 and 10. In Figs. 9a,b, separation using the ''bad'' column (b) is quite different than for the ''good''
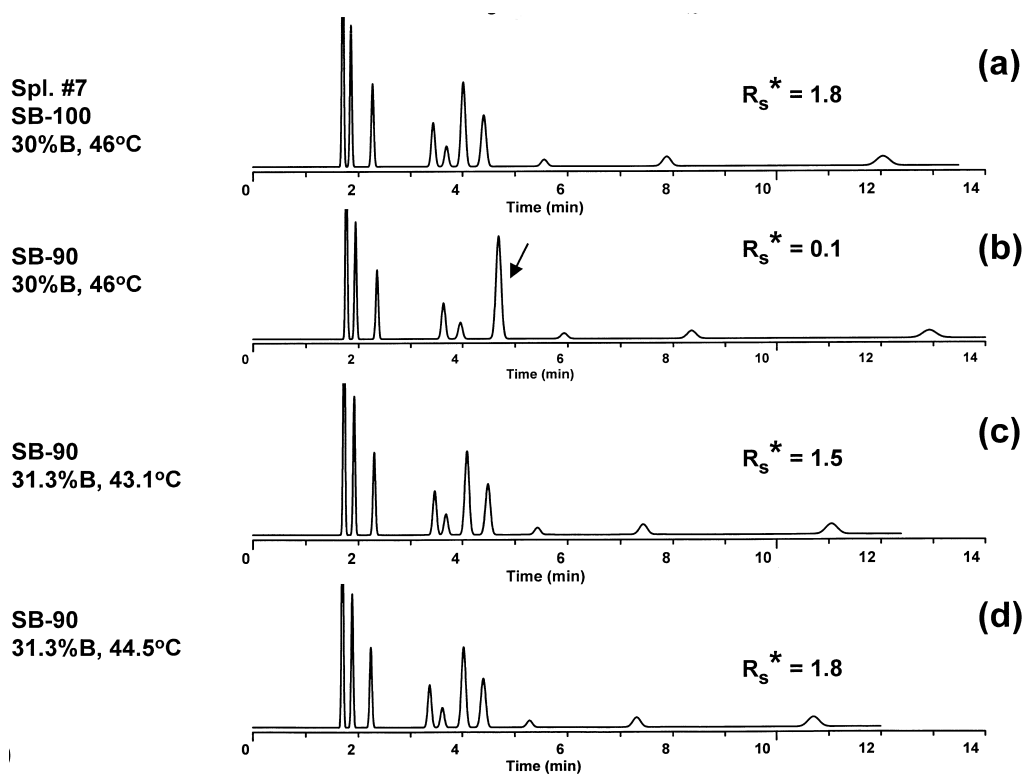
Fig. 8. Method adjustment based on trial-and-error changes in temperature and solvent strength (%B). Sample composed of compounds #21, 23, 31, 40, 41, 42, 44, 46, 65, 67 of Table 1. (a) Separation on "good" column (SB-100); (b) separation on "bad" column (SB-90); (c) adjusted separation based on Eq. (1); (d) adjustment based on further trial-and-error changes in temperature and %B. DryLab 2000 simulations.

column; two bands (#8/9) have reversed positions. Following method adjustment (change in *T* and %B) via Eq. (1) (c), the separation order on "good" and "bad" columns is now the same, but resolution on the "bad" column has been restored only partially ($R_s^* = 1.6$ in [c] vs. 2.2 in [a]). Further trial-and-error adjustment of %B and temperature for this case was unable to increase critical resolution further.

The reason for the relative failure of method adjustment in Fig. 9 can be appreciated from the partial chromatograms of Fig. 10 (non-critical band #10 omitted, to conserve space) for the "bad" column, which represent the adjusted separation (Fig. 10c) of Fig. 9a (same conditions) plus additional runs (a,b,d,e) in which *T* and %B have been changed slightly, to higher or lower values. The arrows in Fig. 10 indicate critical band-pairs; depending on conditions, it is seen that band-pairs

#2/3, 4/5, 7/8 or 8/9 can become critical. As also seen in Fig. 10, the resolution of some of these critical band-pairs varies in opposite fashion as *T* and %B are changed, so that the separation of Fig. 10c cannot be improved by any further adjustment of *T* and/or %B.

4.2.3. Impractical changes in conditions

Occasionally the application of Eq. (1) will suggest values of one or more conditions that are unreasonable; e.g. a change in temperature by hundreds of degrees, negative values of %B, a change in pH of 10–20 units, etc. Alternatively, the recommended changes in some condition may simply appear excessive, for example as judged by the recommendations of [11,12]. In such cases, the condition in question can be changed in the direction recommended by Eq. (1) by a maximum practical
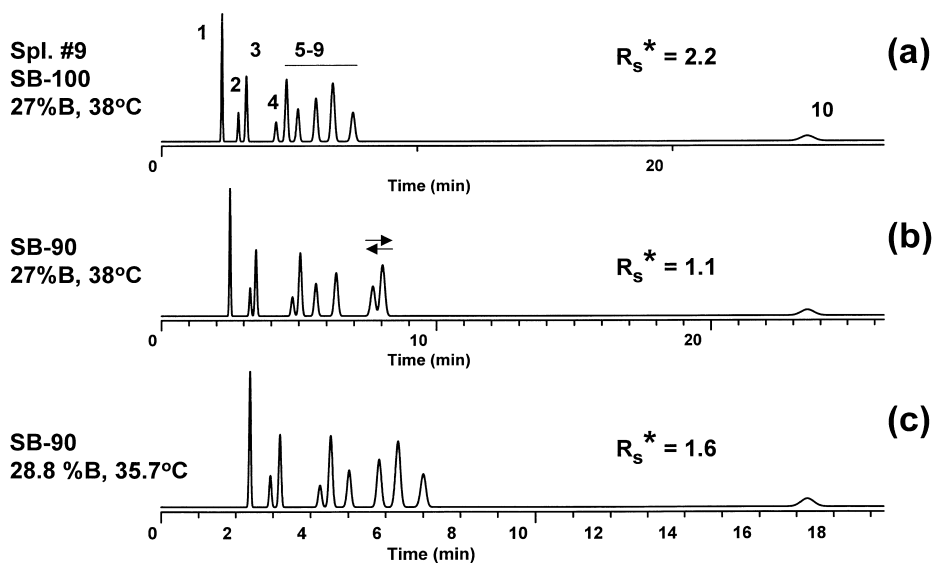
Fig. 9. An example of failed method adjustment. Sample composed of compounds #21, 22, 23, 24, 31, 40, 49, 55, 65, 66 of Table 1. (a) Separation on ''good'' column (SB-100); (b) separation on ''bad'' column (SB-90); (c) adjusted separation based on Eq. (1). DryLab 2000 simulations.
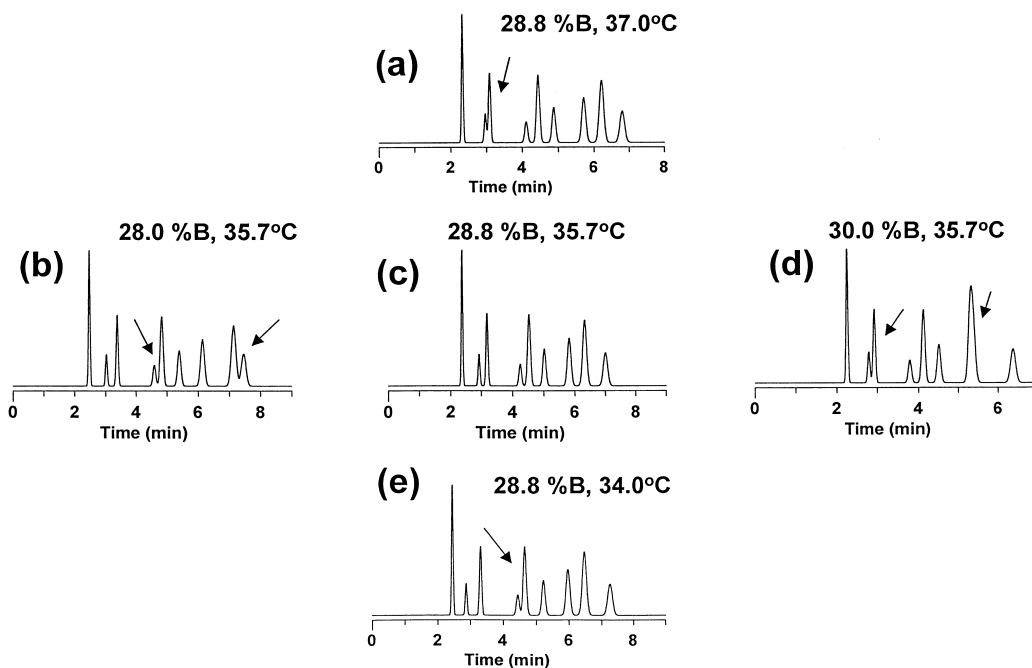

Fig. 10. Separations of sample of Fig. 9 on ''bad'' column as a function of temperature and %B. Same %B for separations a, c and e. Same temperature for separations b, c and d. DryLab 2000 simulations.

amount, followed by reapplication of Eq. (1) while omitting the latter condition from the regression. Alternatively, since such a result suggests only a small effect on resolution of the condition in question, that variable can be eliminated for purposes of method adjustment.

4.3. Other contributions to column variability

The present examples are based on two columns differing in the extent of ligand bonding (see Experimental). A reviewer has pointed out that differences in silica (especially trace metal contamination) are likely to contribute more significantly to batch-to-batch column variability than are differences in bonding. We agree with this assessment, but it should be pointed out that the present "good" and "bad" columns were intended as extreme examples of a difference in ligand concentration. It is therefore likely that these two columns present a similar challenge to method adjustment as would be the case for columns manufactured from slightly different silicas. This conclusion is supported by previous (successful) examples of method adjustment [10] that involved column packings from different sources.

5. Conclusions

A general procedure has been described for a change (adjustment) in separation conditions so as to compensate for batch-to-batch differences in column selectivity. This approach requires that additional ("off-set") separations be carried out, where in each run a single condition is changed—so as to establish the effect of that condition on separation. Conditions that affect isocratic separation selectivity (and are therefore useful for method adjustment) include column temperature and the composition of the mobile phase (%B, pH, buffer or additive concentration, varying proportions of two or more organic solvents). For gradient separations, gradient time t_G replaces isocratic %B. The success of this procedure increases for: (a) samples with a smaller number of components and (b) the use of a larger number of adjustable conditions. If only temperature and %B (or gradient time) are allowed to vary, method adjustment should prove successful for a majority of

samples that contain 14 or fewer components. Method adjustment for samples with a larger number of components is likely to be successful, if conditions in addition to T and t_G are varied. The extension of the present approach to other chromatographic methods which can involve column variability seems logical but has so far not been investigated.

This same method adjustment approach can also be used to correct for changes in column selectivity (but not loss in plate number) as a result of use or column aging. That is, small, simultaneous changes in various conditions can be used to partially restore the original separation on the new column, thereby extending the useful life of the column. Finally, method adjustment as described here can also be used during method development to improve separation, by means of systematic small changes in one or more separation conditions (proposed originally in Refs. [16,20]). The latter method development option should prove more useful, as the number of sample analytes increases.

6. Nomenclature

B	mobile phase B-solvent (acetonitrile in the present study); %B refers to vol.% of B-solvent in the mobile phase
CV	coefficient of variation
$CV(\delta R_s/R_s)$	CV of quantity $(\delta R_s/R_s)$ for all adjacent band-pairs in the sample
n	number of components in a sample
N	plate number
r	correlation coefficient
R_s	resolution of two adjacent bands
R_s^*	"critical" resolution of a chromatogram, equal to R_s for the poorest-resolved band-pair
SB-90, SB-100	designation of two columns used in present study
SE	standard error (Fig. 2)
t_G	gradient time (min)
t_R	retention time (min)
T	temperature (°C)
x, y	variables in equation $y = ax$ (Fig. 2)

x_1, x_2	coefficients of Eq. (1), equal to the required change in each condition for optimal method adjustment
X	a separation condition, e.g. temperature
δR_s	a difference in values of R_s for a given band pair and two columns or for the same column and a change in conditions
ΔX	a change in some condition X

Appendix A

An example of method adjustment

For the example of Fig. 1, we have the values of resolution R_s or change in R_s shown in Table 2.

Next, a multiple regression is carried out of values of $-y$ ("bad" vs. "good" column) vs. *x1* (change in *T*) and *x2* (change in %B) from Table 2. The results of the regression are: $r = 0.990$ (indicating a good correlation and adjustment), $SE = 0.059$ (confirming a good correlation and adjustment), *a1* = 0.560, and *a2* = 0.223. The coefficient *a1* indicates the recommended change in *T*, equal to 0.560 *x* (off-set change in *T*, $+5\,°C$) $= 2.80\,°C$, or an adjusted temperature of $35 + 2.80 = 37.8\,°C$. The coefficient *a2* indicates the recommended change in %B, equal to 0.223 *x* (off-set change in %B, -3%B) $= -0.67$%B, or an adjusted %B $= 50 - 0.67 = 49.3$%B.

Correction for predictive errors

Occasionally, for various reasons, it may be found that the predicted conditions for a successful adjustment yield a separation whose experimental resolution is found to be inadequate. That is, predictions based on the present approach are somewhat in error. This is more likely, the greater the change in adjusted conditions. In such cases, the experimental separation after adjustment of conditions can be used in place of the original (unadjusted) separation on the

Table 2
Values of resolution R_s or change in resolution (δR_s) for various band-pairs of Figs. 1a,b and 3b,c

Column/conditions	Resolution R_s for indicated band-pair			
	#1/2	#2/3	#3/4	#4/5
(a) *Values of R_s*				
"good" column, 50%B, 35 °C	2.54	3.87	4.89	1.89
"bad" column, 50%B, 35 °C	1.5	5.08	4.17	1.08
"good" column, 50%B, 40 °C	5.06	1.21	5.02	4.42
"good" column, 47%B, 35 °C	1.41	5.88	6.65	−0.93
(b) *Values of δR_s*	Change in resolution δR_s for indicated band-pair			
	#1/2	#2/3	#3/4	#4/5
"bad" column, 50%B, 35 °C	−1.04	1.21	−0.72	−0.81
"good" column, 50%B, 40 °C	2.52	−2.66	0.13	2.53
"good" column, 47%B, 35 °C	−1.13	2.01	1.76	−2.82
(b) *Values of $\delta R_s/R_s$*	Change in relative resolution $\delta R_s/R_s$ for indicated band-pair			
	#1/2	#2/3	#3/4	#4/5
(*y*) "bad" column, 50%B, 35 °C	−0.41	0.31	−0.15	−0.43
(*x1*) "good" column, 50%B, 40 °C	0.99	−0.69	0.03	1.34
(*x2*) "good" column, 47%B, 35 °C	−0.44	0.52	0.36	−1.49

''bad'' column and the application of Eq. (1) re-
peated. The resulting usually small changes in con-
ditions are expected to give a predicted separation
that will prove more accurate, and therefore closer to
acceptable.

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