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Computer simulation for the convenient optimization of isocratic reversed-phase liquid chromatographic separations by varying temperature and mobile phase strength

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Abstract

Software is described which allows the rapid development of separations by means of isocratic reversed-phase liquid chromatography (RP-LC), based on the optimization of column temperature (T) and mobile phase strength (%B). For a given sample, four initial experiments are carried out at two different temperatures, using either isocratic or (better) gradient elution. If isocratic experiments are chosen for computer simulation, it is necessary to select appropriate values of %B for these initial runs. Literature data for solute retention as a function of T are reviewed, as a basis for estimating suitable values of %B at the two values of T selected. The use of optimized values of T and %B led to acceptable separations for three representative samples. The prediction of isocratic separation on the basis of initial gradient experiments is more convenient than the use of initial isocratic experiments, but less reliable. When gradient experiments are used, one additional isocratic experiment can improve the accuracy of such predictions by a ''reflection'' procedure. The latter approach was confirmed for predictions of both isocratic and gradient separation from initial gradient experiments. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Computer simulation; Optimization; Column temperature; Mobile phase strength

1. Introduction

Previous work [1–5] has shown that simultaneous changes in temperature (T) and either gradient time (t_G) or solvent strength (%B) can be effective for the control of band spacing, permitting the adequate separation of different samples by means of reversed-phase liquid chromatography (RP-LC). Optimized gradient separations are conveniently developed using computer simulation (DryLab for Windows, Version 2.0; LC Resources) [3–8], by means of which sample resolution can be displayed as a function of T and t_G. Fig. 1 provides an example of such an application, for the separation of a 15-component mixture of basic drugs. Four experimental separations were carried out initially with $T=30$ or 70°C, and $t_G=20$ or 60 min; data from these separations were then used as input for computer simulation. A resolution map as a function of T and t_G was requested (Fig. 1a; note cross-hairs marking maximum resolution), and the resulting optimized separation for $T=51$°C and $t_G=76$ min was predicted (Fig. 1b). Predictions of gradient separation from gradient data, as in Fig. 1, have been found to be quite reliable [9,10].

While a gradient method can be developed for any sample, isocratic separation is often preferred. Isocratic RP-LC separations as a function of T and %B

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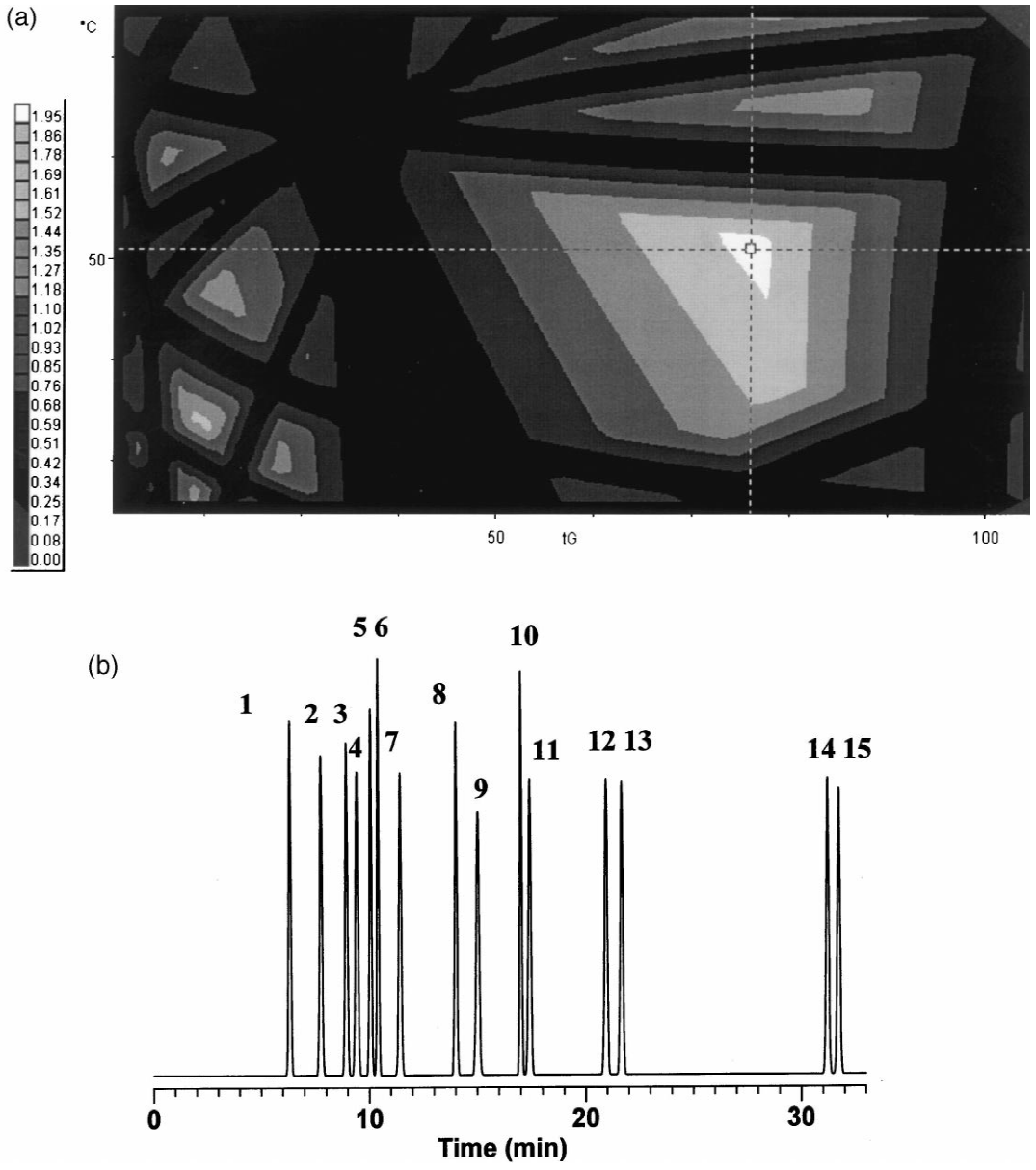


Fig. 1. Computer simulations for the separation of a 15-component mixture of basic toxicology standards. The sample excludes ''pre-eluting'' compounds ≠1–7 of the 22 basic drugs listed in Table 3 of [9]. (a) Resolution map as a function of temperature and gradient time, based on 0–100% *B* gradients in 20 and 60 min, for $T=30$ and 70°C; (b) optimized separation selected from map of (a); 0–100% acetonitrile–buffer gradient in 76 min; 51°C; 25×0.46 cm C_{18} column; 2.0 ml/min. For other conditions, see [9]. Cursor (cross hair) in (a) marks conditions for maximum resolution of the sample.

can be predicted on the basis of four initial experiments (as in Fig. 2a), using either isocratic or gradient runs where T and %B or t_G are varied – similar to the procedure that led to the separation of Fig. 1b. The use of initial gradient experiments is more convenient, for reasons discussed in the present paper, but isocratic predictions from gradient data tend to be less reliable [10]. While previously

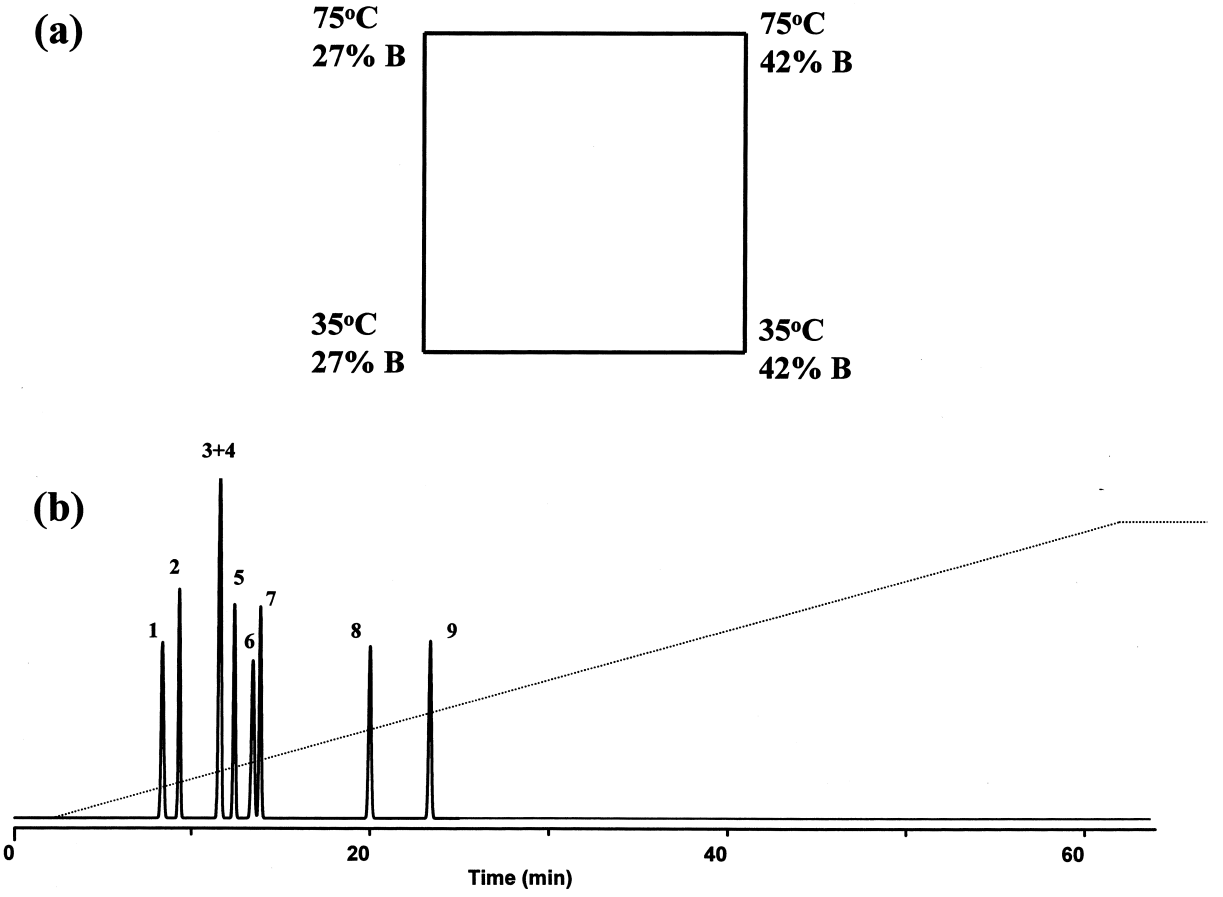


Fig. 2. Illustration of the present experimental approach: selection of conditions for the initial four runs. Separation of a 9-component sample composed of substituted benzoic acids and anilines. (a) Experimental design, with indicated temperatures and %*B* values for each of the four initial experiments; (b) initial gradient separation for the purpose of estimating best conditions in Fig. 2a; 5–100% acetonitrile/pH 2.6 buffer gradient in 60 min; 2.0 ml/min; 35°C; other conditions as in Experimental section; (c–h) isocratic separations for indicated conditions of temperature and gradient time, other conditions as in (b). Conditions for (g,h) selected as described in Appendix A. These separations (b–h) are each computer simulations based on four initial gradient runs: 5–65% *B*, 37.6 and 76.8°C, gradient times of 13 and 39 min. Other conditions as in [10]. Sample: (1) 3ClA; (2) 2FB; (3) 2ClB; (4) 3NB; (5) 3FB; (6) 2ClA; (7) 26DMB; (8) 34DCA; (9) 35DCA.

reported software (DryLab, version 2.0) for optimizing T and t_G as in Fig. 1 [3–8] allows the prediction of isocratic separations from gradient data, it does not provide (more convenient) resolution maps for corresponding isocratic separations (R_s versus T and %B). The present paper describes the use of a software upgrade (DryLab 2000 for Windows) that overcomes these prior limitations of computer simulation for the optimization of isocratic separations where T and %B are varied. The advantages and limitations of this approach for developing an isocratic RP-LC procedure are also explored.

2. Theory

2.1. Retention predictions for varying temperature and mobile phase strength (%B)

The variation of retention k with %B in RP-LC is usually well represented [11] by

$$\log k = \log k_w - S\phi \quad (1)$$

where k is the retention factor of a solute, k_w is the extrapolated value of k in water (0% B), S is an empirical constant for a given solute and only %B

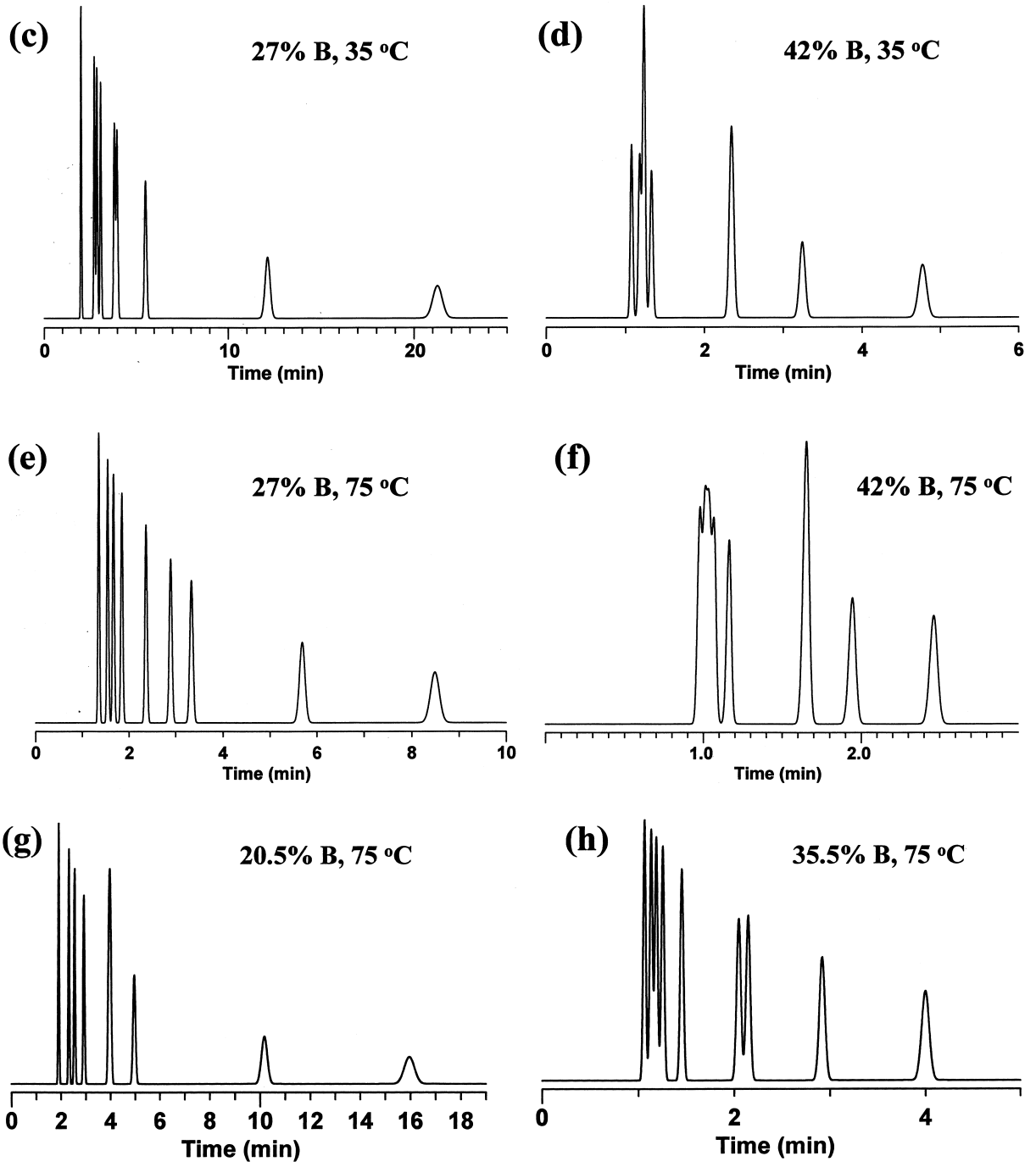


Fig. 2 (*continued*).

varying, and ϕ (equal 0.01% *B*) is the volume fraction of the B-solvent in the mobile phase. Similarly, *k* varies with absolute temperature T_K as

$$\log k = A + B/T_K \quad (2)$$

If measurements of *k* are made at two different values of *T* and ϕ (four runs total, see example of Fig. 2a), it is possible to predict values of *k* as a function of *T* and ϕ. Thus, Eq. (1) allows the determination of values of log k_w and *S* for each temperature (e.g., 35 and 75°C as in Fig. 2a), which then permits the prediction of *k* at these two temperatures for any value of %*B*. The coefficients *A* and *B* of Eq. (2) can then each be determined as functions of %*B*, which allows predictions of *k* as a function of *T* for any value of %*B*. The plate number *N* for each peak in the sample can be predicted as a function of separation conditions [12], so that computer simulation also allows the prediction of band width (and resolution) for changes in *T*, %*B*, column dimensions, particle size and flow-rate.

Retention in isocratic and gradient elution can be compared in terms of retention factors *k* (isocratic) or *k** (gradient) [13], where

$$k^* = 0.85 \cdot t_G \cdot F/(V_m\, \Delta\phi\, S) \quad (3)$$

F is flow-rate, V_m is column dead volume, and $\Delta\phi$ is the change in ϕ during the gradient. Given a range in *k** for the initial gradient experiments ($4<k^*<12$ in the present study), predicted values of *k* for isocratic separation will be more reliable for interpolation ($4<k<12$) than for extrapolation ($4>k>12$) [10].

2.2. Correcting errors in initial predictions by computer simulation

Errors in computer simulation can occur for various reasons [10], but a common cause of such errors is a failure of Eqs. (1) or (2). Further errors are introduced when gradient data are used to predict isocratic separations. We have proposed a general approach [10] for correcting all such errors, the use of a so-called ''reflection'' technique. This procedure is illustrated in Fig. 3, where error in isocratic predictions from gradient data (*T* constant) is considered. Fig. 3a shows a series of runs where only %*B* varies. Maximum resolution ($R_s = 1.7$) is predicted for a mobile phase of 11% *B*, but the corresponding experimental separation (Fig. 3b) is unacceptable – peaks 4ClA and 3CB overlap completely. The error in this prediction can be expressed in terms of an equivalent error in %*B*, which by comparison of Figs. 3a and b appears to be about +1% *B*. Thus, the experimental run for 11% *B* (Fig. 3b) appears to closely match the predicted separation for 12% *B* in Fig. 3a; i.e., the predicted value of %*B* is 1% high. Note that our comparisons of these separations emphasize resolution; 5–10% differences in retention time apart from resolution are regarded as less significant.

2.2.1. Correction of predictions by a ''reflection'' procedure

Having established that computer simulations in the region of optimum conditions (11% *B* predicted) are in error by +1% *B*, we conclude that the true optimum must occur for 10% *B*. The actual separation for the latter conditions (Fig. 3c) closely matches our originally predicted optimum (11% *B* in Fig. 3a). The process of arriving at the corrected prediction of Fig. 3c consists of, (a) a determination of the ''error'' in conditions (+1% *B* in this example) by a matching of experimental (Fig. 3b) versus predicted (Fig. 3a) separations, followed by, (b) a subtraction or ''reflection'' of this error to arrive at the correct final prediction. This ''reflection'' procedure will usually be successful for a single critical band-pair in the sample, or when two critical band-pairs form a triplet (as in Fig. 3). When additional critical bands are important, correction by ''reflection'' may prove only moderately successful. However, the extent of correction is expected to improve when more than one variable is used to optimize separation; e.g., simultaneously optimizing *T* and %*B* as described here. In the latter case, correction of computer simulation can address errors in both *T* and %*B*. That is, the reflection procedure can involve adjustment in both *T* and %*B* [10], as further discussed in this paper.

3. Experimental

The present study is based on computer simulations carried out using DryLab 2000 for Windows

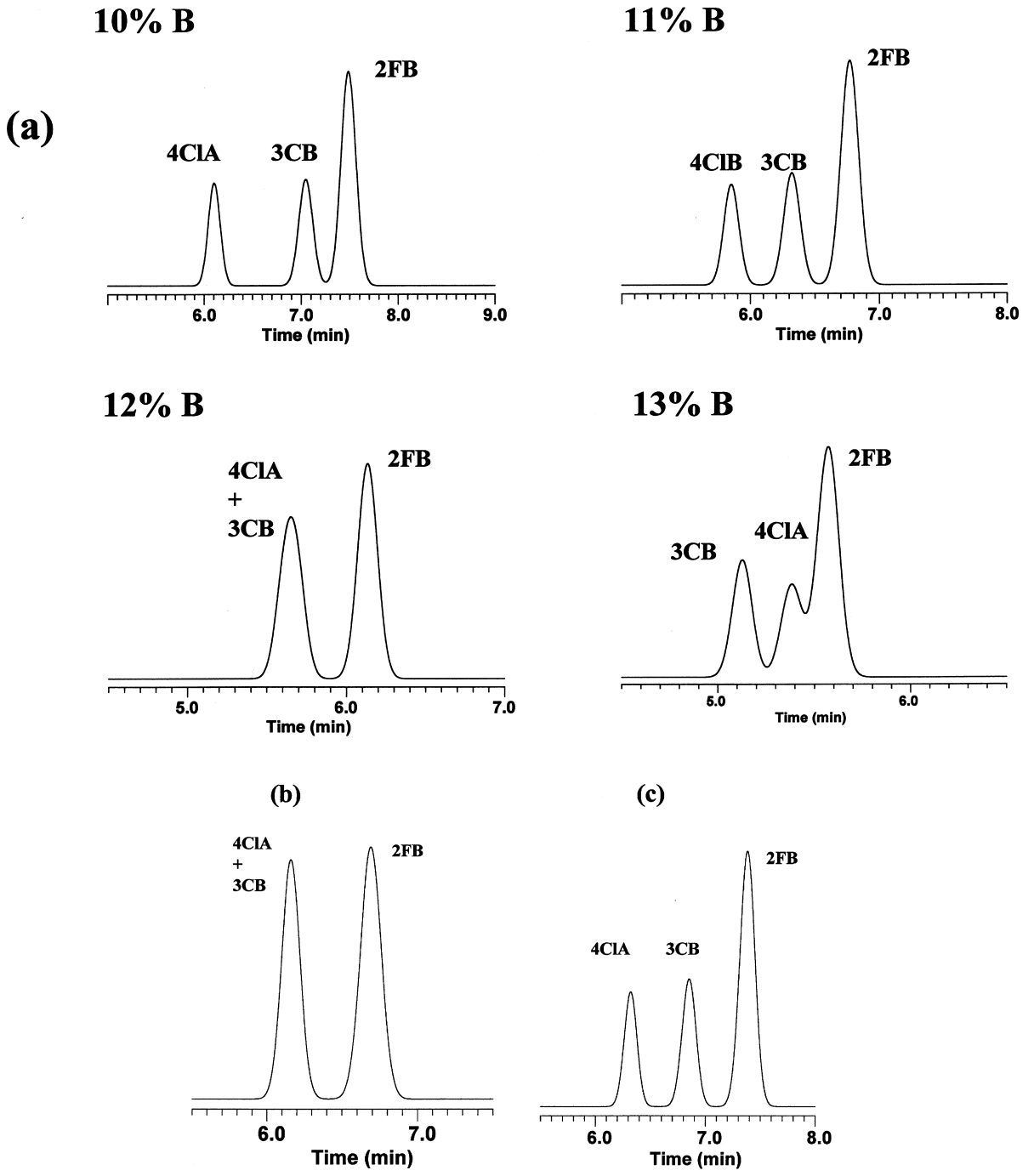


Fig. 3. Correction for errors in computer simulation by means of the ''reflection'' procedure. Separation of a 3-component mixture; conditions as in Experimental section unless noted otherwise. (a) Computer predictions of separation, based on four gradient input runs; (b) experimental run for 11% *B* (predicted optimum conditions); (c) experimental run for 10% *B* (actual optimum conditions). See text for details.

(LC Resources). Gradient (input) data required for these simulations are experiments that were reported previously [10] for a 14-component mixture of substituted benzoic acids and anilines. The sample consisted of the following compounds: phthalic acid (PA), 2-nitrobenzoic acid (2NB), 3-nitrobenzoic acid (3NB), 3-cyanobenzoic acid (3CB), 2-fluorobenzoic acid (2FB), 3-fluorobenzoic acid (3FB), 2-chlorobenzoic acid (2ClB), 2,6-dimethylbenzoic acid (26DMB), 3,5-dimethylaniline (35DMA), 2-chloroaniline (2ClA). 3-chloroaniline (3ClA), 4-chloroaniline (4ClA), 3,4-dichloroaniline (34DCA) and 3,5-dichloroanilne (3,5DCA). Conditions were as follows: 15×0.46 cm C_{18} column (Zorbax SB-C_{18}; Hewlett-Packard); A-solvent, pH 2.6, 50 m*M* phosphate buffer; B-solvent, acetonitrile; 1.5 ml/min. Nine 5–65% *B* gradient runs were carried out at temperatures of 37.6, 56.7 and 76.8°C, in gradient times of 13, 26 and 39 min. Unless otherwise noted, four runs with $T=37.6$ and 76.8°C and $t_G=13$ and 39 min were used as input for computer simulation, and the gradient range was always 5–65% *B*. The equipment dwell volume was 2.3 ml. Additional isocratic experiments were carried out for the present study, using the same equipment and similar conditions.

3.1. Computer simulations

One goal of the present study was a test of the accuracy of computer predictions of resolution R_s for the present sample and different conditions, especially of isocratic predictions from gradient data when both %*B* and *T* are varied. Predictions of resolution and of final chromatograms also require values of the bandwidth *W* of each peak in the chromatogram. The present software allows predictions of *W* based on either, (a) interpolations of experimental bandwidths from the input experiments as in Fig. 2a, (b) use of a single column plate number *N* for all bands and conditions, or (c) a calculated value of *N* for each band and set of conditions (described in [12]). Predicted chromatograms and resolution maps displayed in Figs. 1–9 use option (c) as a basis for calculations of *W*. ''Experimental'' chromatograms shown in various figures are computer recreations based on experimental values of t_R with calculated values of *W* (option c), which means that comparisons of resolution between experimental and predicted separations are not affected by uncertainty in bandwidths. In Fig. 9, ''actual'' chromatograms are simulations based on the use of input experiments which have values of *T* (57, 76°C) and t_G (26 and 39 min) which are not too different, conditions which have been shown [10] to minimize error in predicted values of t_R. In the latter case, ''actual'' and experimental separations for the same conditions become essentially equivalent.

4. Results and discussion

4.1. Computer simulations based on initial isocratic experiments

4.1.1. Selection of initial values of T and t_G

For use of the experimental design of Fig. 2a, it is necessary to first select values of *T* and %*B*. Values of *T* are easily determined; for most HPLC equipment, it is desirable to choose the lower temperature to be at least 5°C above ambient; e.g., 30–35°C. The higher temperature should allow the exploration of as wide a temperature range as possible, thereby increasing the likelihood of finding conditions for adequate resolution of the sample. We recommend a temperature difference of 30–40°C; in the example of Fig. 2a, values of $T=35$ and 75°C were selected.

The choice of %*B* values cannot be made arbitrarily, since for isocratic separation it is desirable that $0.5\leq k\leq 20$. There are two approaches for determining appropriate values of %*B* as in Fig. 2a [14]. First, trial-and-error changes in %*B* can be explored at the lower temperature (35°C), eventually allowing a choice of suitable %*B* values for the two runs at this temperature. However, this approach may require several experiments. Alternatively, an initial gradient elution run at 35°C can be carried out, which allows suitable values of %*B* in Fig. 2a to be estimated without the need for further experiments (p. 362 of [13]). The latter approach is illustrated in Fig. 2b, for a mixture of nine substituted benzoic acids and anilines selected from the sample compounds described in the Experimental section. Gradient time $t_G=60$ min, and other conditions (15×0.46 cm C_{18} column, 2.0 ml/min flow-rate, 5–100% acetonitrile/buffer gradient, 35°C) are recommended (p. 362 of

[13]). Whereas several trial-and-error isocratic experiments may be required in the first approach, the use of a single gradient run as in Fig. 2b suffices in the second approach (which is therefore preferred). Thus, from Table 8.2 of [14], we find that 27% *B* (at 35°C) should give a value of $k \approx 20$ for the last peak (Fig. 2b, $t_R = 24$ min); a value of $k = 23$ was actually observed (Fig. 2c). This %*B* value is selected for maximum retention, so the second %*B* value of Fig. 2a must be >27% *B*. Typically, for computer simulation based on experiments as in Fig. 2b, %*B* values differ by 10–20% *B* (see further discussion in [10]). At this point, we arbitrarily choose a difference of 15% *B*, so that resulting values of %*B* are 27 and 42%. These runs are shown in Figs. 2c–f.

A possible problem – early elution at the higher temperature – can arise in the selection of these particular run conditions (Figs. 2e and f). When varying *T* and %*B*, it is desirable to maintain $0.5 < k < 20$ for all bands, but this is complicated by the dependence of values of *k* on *both T* and %*B*. Because retention generally decreases at higher temperatures, early bands in the run with 42% *B* and 75°C are bunched up and poorly resolved. This will make the accurate determination of values of t_R for these initial bands difficult, in turn compromising the reliability of computer simulation predictions.

One solution to this problem is to select lower values of %*B* at the higher temperature, such that the range in values of *k* at 75°C is similar to that of Figs. 2c and d at 35°C. For this approach, we require an estimate of how retention changes with temperature. The discussion of Appendix A allows a basis for estimating the effect of temperature on retention, and provides rules for adjustments in %*B* so as to compensate for decreased retention at higher *T*. A simplified procedure is to reduce %*B* by 0.16% for each 1°C increase in *T*. Figs. 2g and h show resulting separations with adjustment of %*B* values in this way. The range in t_R and *k* for these latter two experiments is seen to be similar to that for the two runs at 35°C (Figs. 2c and d), with less crowding of early peaks in Fig. 2h versus 2d. Note, however, that the dependence of *k* on *T* depends somewhat on the nature of the sample and separation conditions (see Appendix A). The prediction of retention versus temperature and resulting adjustments in %*B* (as in Figs. 2g and h) may therefore prove less reliable for individual cases.

4.2. Isocratic predictions based on initial gradient experiments

Predictions of isocratic separation as a function of *T* and %*B* can be carried out using computer simulation and the experimental design of Fig. 2a. However, if the four isocratic experiments of Fig. 2a are replaced by corresponding gradient runs, where two values of gradient time t_G replace corresponding isocratic %*B* values, it is possible to carry out the optimization of isocratic separation more conveniently. From four initial gradient experiments (e.g., $T =$ 35 and 75°C, $t_G = 20$ and 60 min), the computer can determine values of *S* and k_w for each solute at the two initial (input) temperatures (e.g., 35 and 75°C) [13]. This then permits the prediction of isocratic retention as a function of *T* and %*B* from gradient data (see the Theory section), as for the case where initial isocratic experiments are employed (Fig. 2a). Values of t_G can be selected by means of Eq. (3), which allows any desired range in k^* (and *k*) to be chosen. The use of four initial gradient runs as a basis for optimizing isocratic separation as a function of *T* and %*B* has two advantages when compared to the use of initial isocratic experiments. First, fewer experiments will be required in most cases. Second, the more even spacing of bands in gradient versus isocratic elution usually makes the measurement of gradient retention times more accurate, which in turn results in more reliable predictions of separation by computer simulation.

To illustrate the effectiveness of the latter approach for isocratic method development (based on four initial gradient runs), the 14 substituted benzoic acids and anilines of the Experimental section were sub-divided into three 6-component samples (''Groups'' 1, 2 and 3) as defined in Table 1. Each of the latter three samples can be separated isocratically with $0.5 < k < 20$, whereas this is not true for the entire 14-component sample. For each of the three samples of Table 1, four input runs were carried out with $T = 37.6$ and 76.8°C and $t_G = 13$ and 39 min. Resolution maps were then requested, followed by selection of optimized conditions for the separation.

Table 1
Samples used for isocratic method development in the present study

Sample	Components[a]	Comments[b]
"Group 1"	3FB, 26DMB, 3ClA, 2ClA, 34DCA, 35DCA	strong retention
"Group 2"	2FB, 3NB, 2ClB, 3ClA, 4ClA, 3FB	intermediate retention
"Group 3"	PA, 2NB, 35DMA, 4ClA, 3CNB, 2FB	weak retention

[a] See Experimental section for abbreviations.
[b] For separation at pH 2.6.

The accuracy of these predicted separations was evaluated, and if necessary the predictive accuracy was improved as described in a following section ("Correction of Initial Predictions...").

4.2.1. Separation of "Group 1" sample

A resolution map for this sample is shown in Fig. 4a. The %*B* range on the *x*-axis is scaled to 20–45 %*B* in order to exclude data which fall outside the desired range of $0.5 \leq k \leq 20$; a similar scaling is used in the following resolution maps of Figs. 5a and 6a. Maximum sample resolution ($R_s = 5.4$) is predicted in the vicinity of 52°C and 29% *B* (see cursor, cross-hairs in Fig. 4a). This separation is predicted to be reasonably robust so far as unintended changes in *T* or %*B* (Fig. 4a); the predicted chromatogram for these conditions is shown in Fig. 4b and compared with the actual separation in Fig. 4c ($R_s = 4.5$). The agreement between Figs. 4b and c appears reasonable in terms of the usual goals of method development.

4.2.2. Separation of the "Group 2" sample

A resolution map for this sample is shown in Fig. 5a. Maximum sample resolution ($R_s = 2.5$) is predicted in the vicinity of 20% *B* and 81°C. A comparison of predicted versus actual separations of the group-2 sample for a different set of conditions (18% *B* and 76°C; see cursor in Fig. 5a) is shown in Figs. 5b and c. The latter conditions are intentionally less robust and therefore more subject to predictive errors, thereby providing a more severe test of the accuracy of computer simulation. For the conditions of Figs. 5c and d, the predicted and experimental chromatograms agree about as well as could be hoped, with comparable values of resolution ($R_s =$ 1.9 for both c and d). While robust conditions improve the likelihood of more accurate predictions of separation, it appears that non-robust conditions do not necessarily mean unacceptable predictive errors.

4.2.3. Separation of the "Group 3" sample

A resolution map for this sample is shown in Fig. 6a. Maximum sample resolution ($R_s = 2.7$) is predicted for $T = 76$°C and 12% *B*; errors of ±2°C or ±1% *B* still result in $R_s \geq 2.5$ (robust separation). Fig. 6b shows the predicted separation for these conditions, which provides a further example of the power of the present approach for developing acceptable isocratic separations.

A more critical test of the accuracy of computer prediction for the group-3 sample is provided by conditions (less robust, smaller R_s) other than those of Fig. 6b. Figs. 6c and d show one (especially poor) comparison of predicted versus actual separations for this group-3 sample (64°C, 11% *B*). The predicted resolution (Fig. 6c) was $R_s = 1.7$, while the actual resolution (Fig. 6d) was $R_s = 0$ (complete overlap of bands 4ClA with 3CB). A following section describes the correction of this prediction by the "reflection" technique. It should be kept in mind, however, that (normally preferred) conditions which favor increased robustness and resolution tend to minimize the consequences of predictive errors.

4.2.4. Quantitative description of predictive errors

A previous report [10] has examined likely errors in computer simulation and concluded that predictions of isocratic separation based on gradient input data are less reliable, compared to the use of isocratic input data. Small errors in retention (*k* or t_R) are generally acceptable, but errors in resolution are more important. The study of [10] defined errors in *k* in terms of equivalent errors in %*B* or ϕ ($\delta\phi$), with corresponding errors in R_s equal to differences

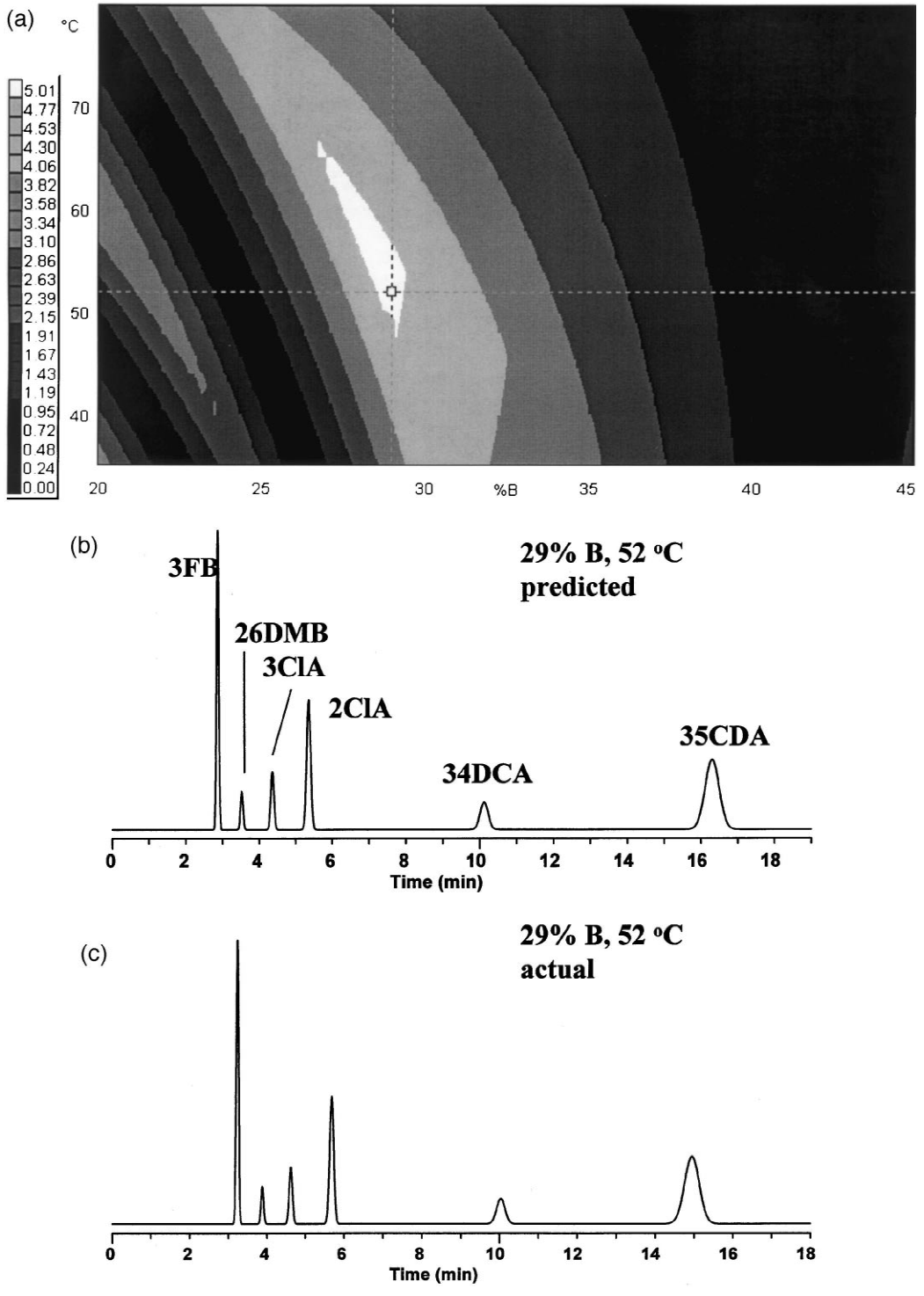


Fig. 4. Computer simulations for the separation of the group-1 sample. (a) Resolution map as a function of T and %B; (b) predicted separation for preferred conditions (52°C, 29% B); (c) experimental separation for conditions of (b).

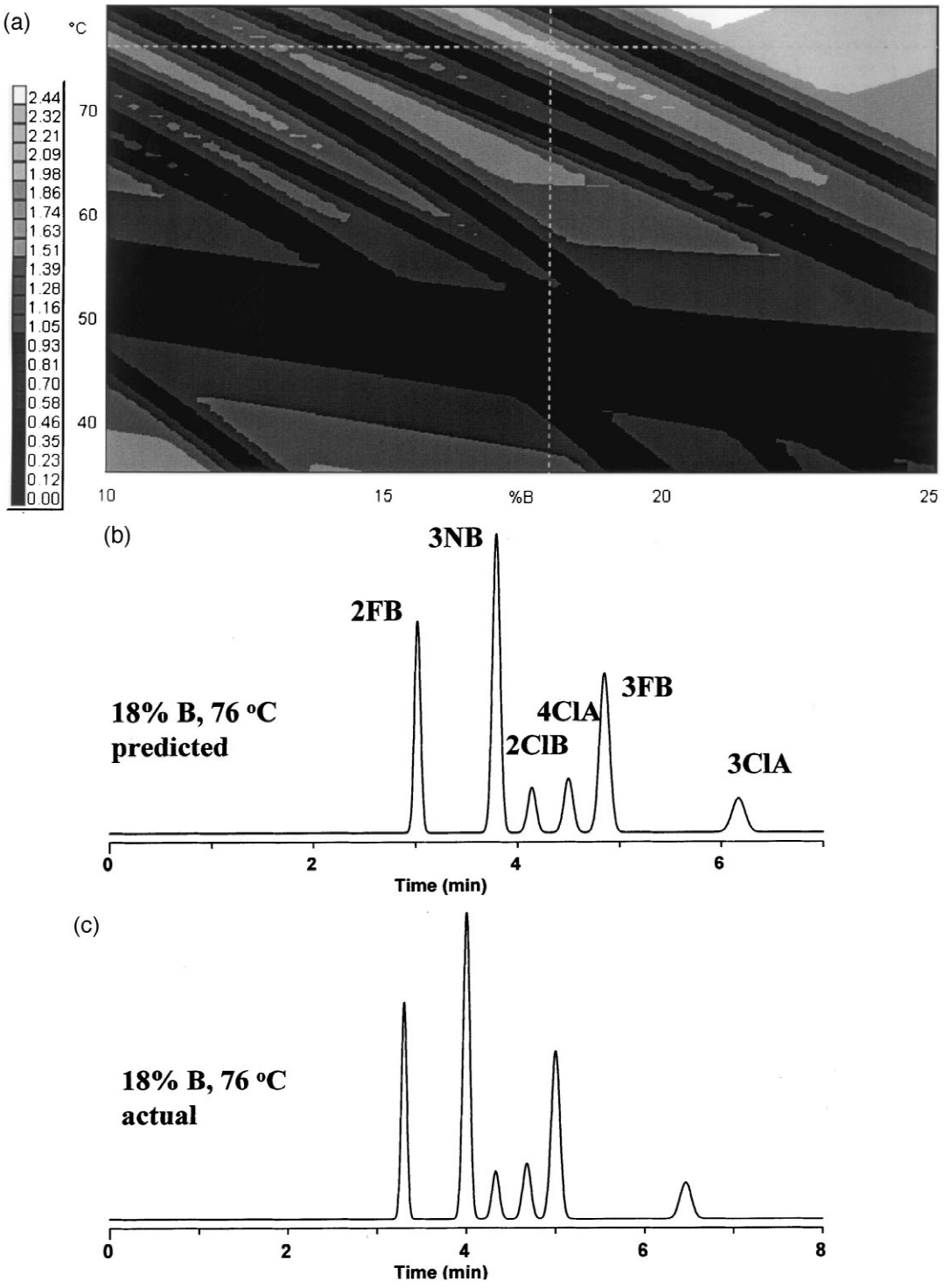


Fig. 5. Computer simulations for the separation of the group-2 sample. (a) Resolution map as a function of T and $\%B$; (b) predicted separation for less robust conditions (76°C, 13% B); (c) experimental separation for conditions of (b).

($\delta\delta\phi$) in $\delta\phi$ for two adjacent bands. That is, a change in the mobile phase by an amount $\delta\phi$ would yield an experimental value of k that equals the value of k originally predicted by computer simulation. Similarly, a change in the mobile phase by an amount $\delta\delta\phi$ for one of the two bands would yield an experimental value of α that agrees with that originally predicted for a given band-pair.

For acceptable predictions, it was concluded [10] that average errors $|\delta\delta\phi|$ (absolute values) for any computer simulation should be ≤ 0.002 units in $|\delta\delta\phi|$, corresponding to typical errors in $R_s \leq 0.4$ units. Table 2 summarizes calculations of $\delta\phi$ and $|\delta\delta\phi|$ for the (poorly predicted) separation of Fig. 6c and d; it is seen that the average value of $|\delta\delta\phi|$ equals 0.007, equivalent to an average error in R_s of 1.4 units. The poor agreement of Figs. 6c and d is hence not surprising. Several predicted separations of the group-1, -2 and -3 samples were compared with experimental runs for conditions similar to those of Figs. 4c, 5b and 6c. A summary of these comparisons of error in predicted separation (calculated as in Table 2) is given in Table 3. The average value of $|\delta\delta\phi|$for the various isocratic separations of Table 3 is ± 0.005 (corresponding to an average error in predicted resolution of ± 1.0 R_s-unit); this can be compared with an average error of $|\delta\delta\phi| = 0.001$ (i.e., ± 0.2 R_s units) for predictions of *gradient* separation as a function of T and t_G [10] (initial gradient runs in each case).This suggests that predictions of isocratic separation (T and %B varying) from gradient data will often benefit from correction; e.g., using the ''reflection'' procedure as described next.

4.3. Correction of initial predictions of isocratic separation (gradient input data)

4.3.1. Isocratic predictions

A poor prediction of the separation of the group-3 sample (for less robust conditions) was observed in Fig. 6 (cf. c versus d). We can apply the ''reflection'' procedure to correct for the errors observed in this initial prediction. The first step is to review *predicted* separations for conditions similar to those initially recommended as optimum (64°C, 11% B). Fig. 7a summarizes predictions which differ from the target (predicted optimum) values (64°C, 11% B) by ± 1°C

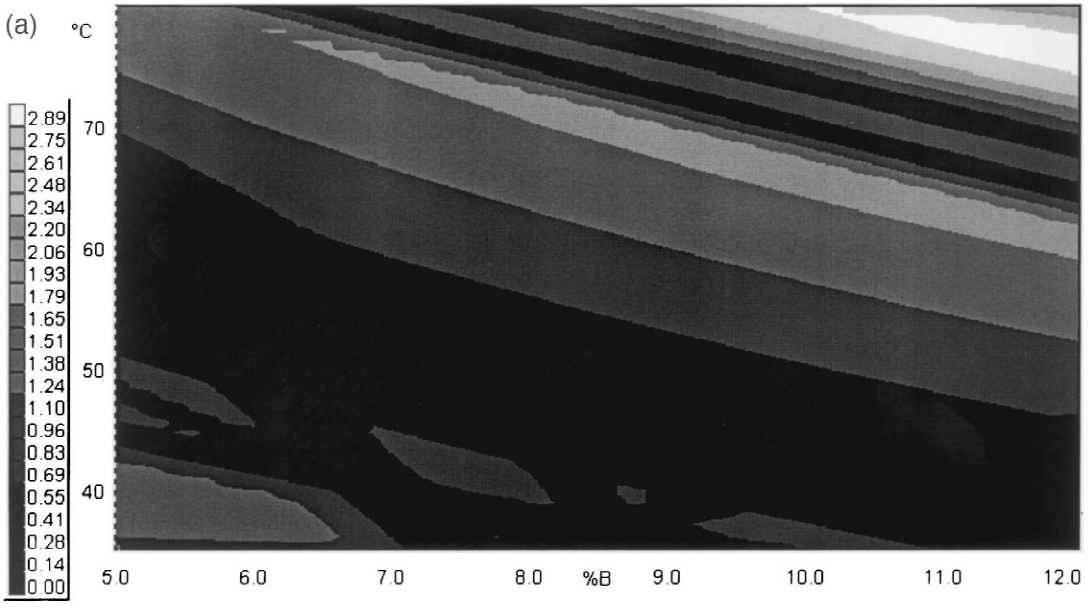


Fig. 6. Computer simulations for the separation of the group-3 sample. (a) Resolution map as a function of T and %B; (b) predicted separation for preferred conditions (76°C, 12% B) (c) predicted separation for other (less robust) conditions (64°C, 11% B); (d) experimental separation for conditions of (c).

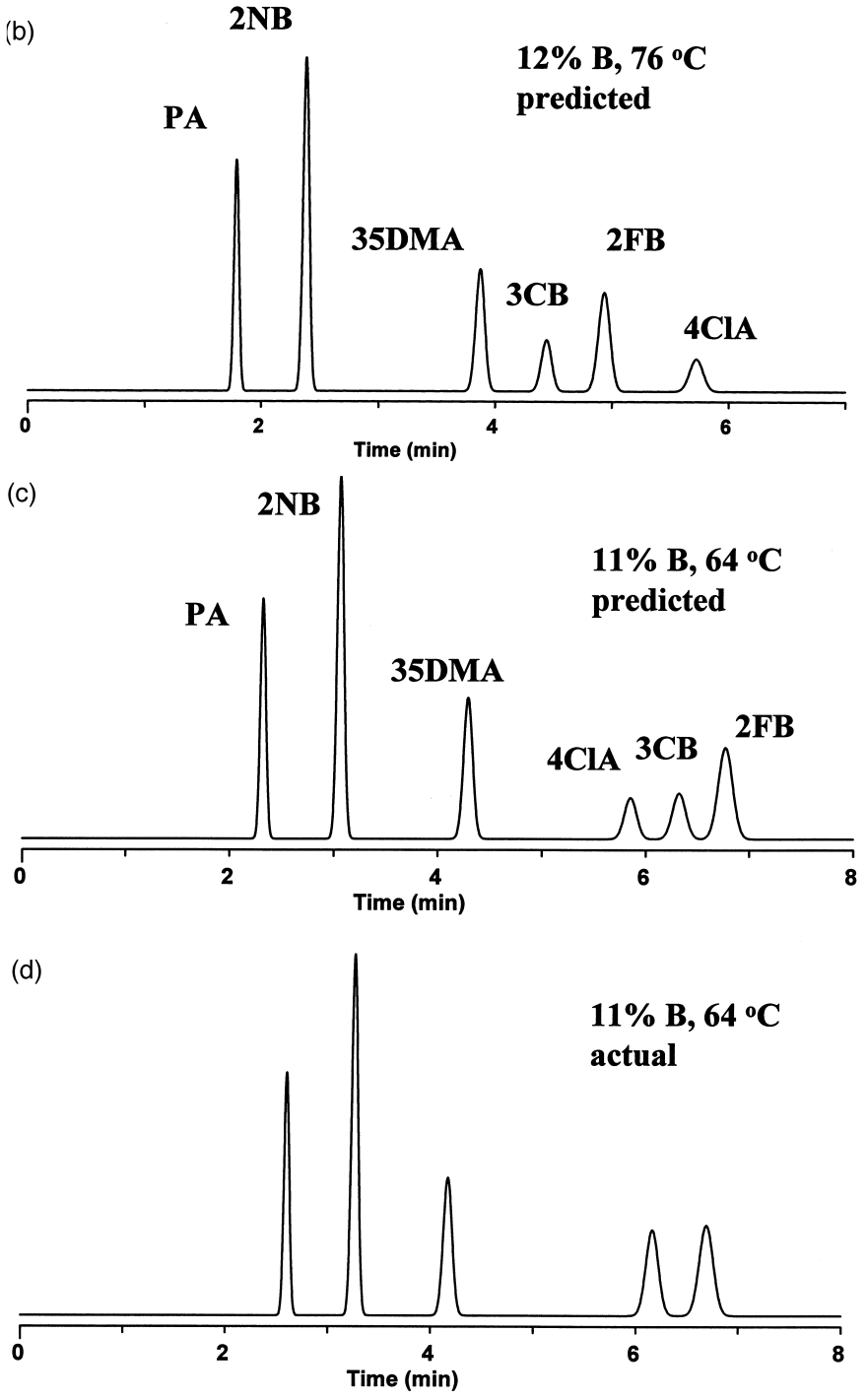


Fig. 6 (*continued*).

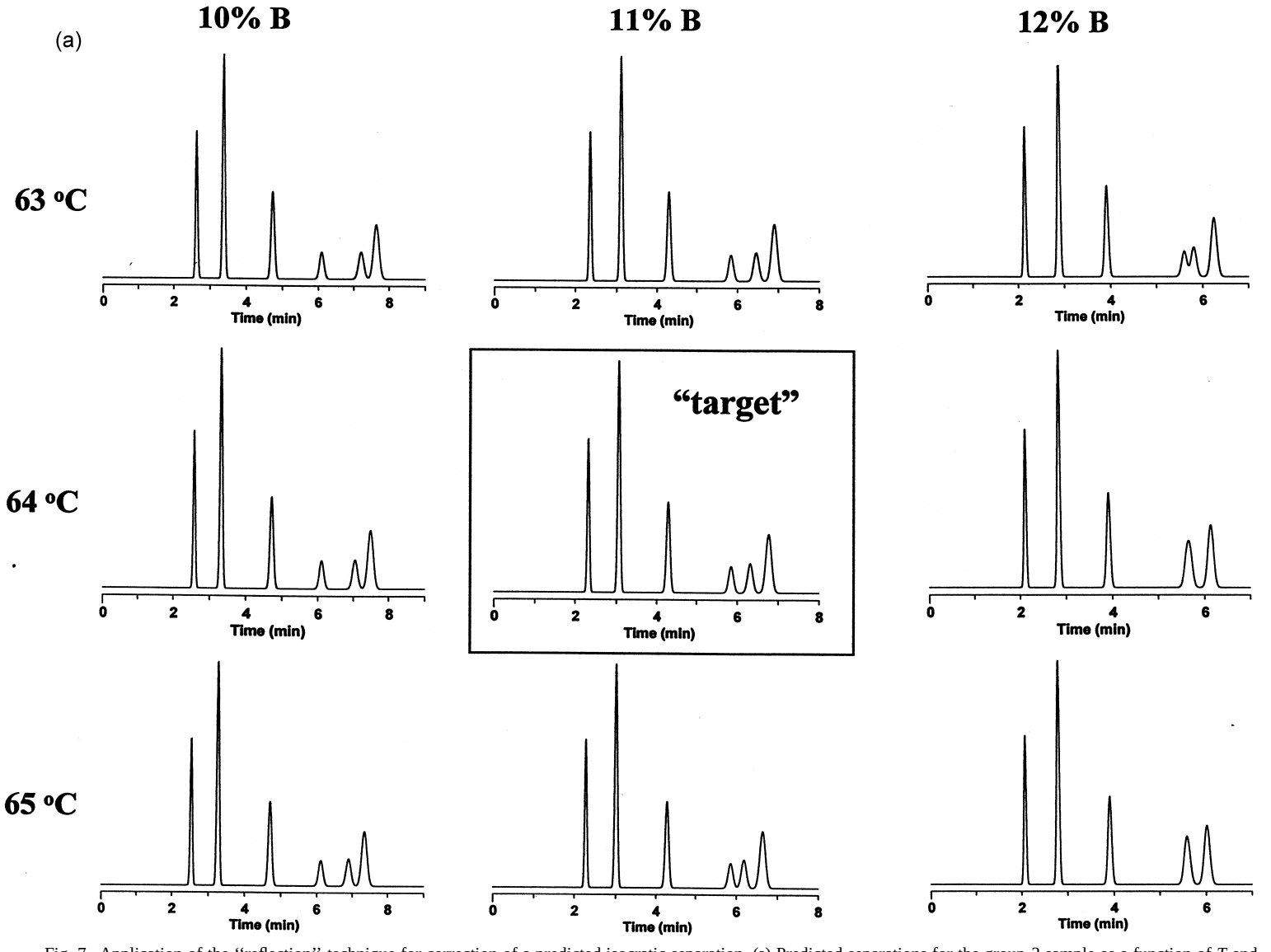


Fig. 7. Application of the ''reflection'' technique for correction of a predicted isocratic separation. (a) Predicted separations for the group-3 sample as a function of T and %B; ''target'' separation is for maximum (optimum) resolution; (b) experimental optimized separation for 64°C and 10% B (corrected by ''reflection'' procedure). See text for details.

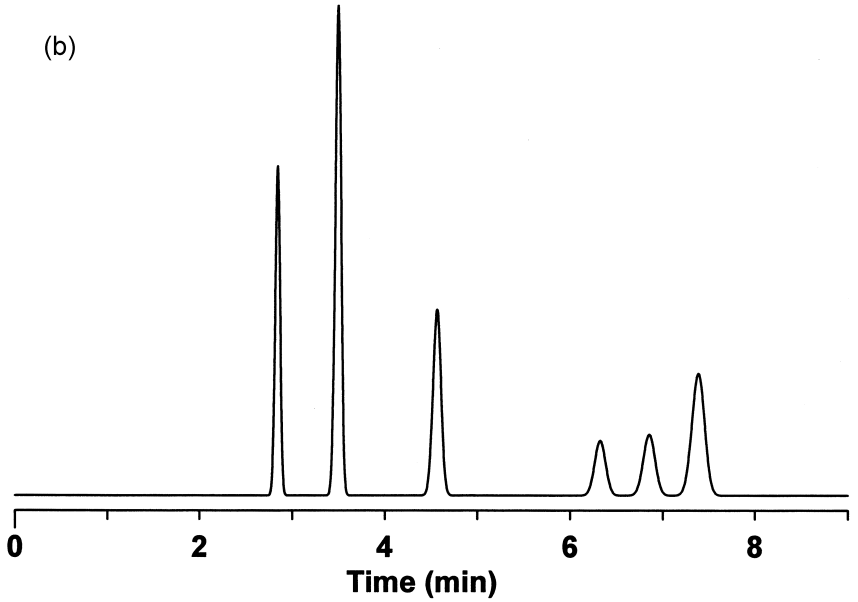


Fig. 7 (*continued*).

and/or ±1% *B*. Comparing these various predictions with the experimental separation of Fig. 6d, we can conclude that runs for 12% *B* and either 64 or 65°C provide a reasonable match. This suggests errors in the conditions for the predicted separations of +1% *B* and 0 or 1°C. Arbitrarily choosing no error in *T*, the corrected conditions (by ''reflection'') for optimized separation are 64°C and 10% *B*. The experimental run for these conditions (Fig. 7b, R_s = 2.0) is in reasonable agreement with the original (target) prediction in Fig. 6c (R_s = 1.7).

Table 4 provides a further comparison of the target

Table 2
Comparison of predicted and actual separations of the group-3 sample for 11% *B* and 64°C

Solute	Retention time (min)		Error $\delta\phi$[a]	Error $\lvert\delta\delta\phi\rvert$[a]	k[b]
	Predicted	Actual			
PA	2.32	2.60	−0.016	0.008	1.5
2NB	3.06	3.27	−0.008	0.010	2.1
35DMA	4.29	4.18	0.003	0.008	3.0
4CLA	5.85	6.16	−0.005	0.007	4.9
3CB	6.32	6.16	0.002	0.001	4.9
2FB	6.77	6.7	0.001	–	5.4
average				0.007	

[a] Values calculated as described in [10]; see text for details.
[b] Isocratic predictions for $4<k<12$ are interpolations and therefore more reliable; the average error $\lvert\delta\delta\phi\rvert$ for interpolations is ±0.004, versus ±0.009 for extrapolations ($k<4$).

Table 3
Summary of errors $\lvert\delta\delta\phi\rvert$ found in the prediction of isocratic separation from gradient input data for the samples of Table 1

Sample	Conditions		Avg. $\lvert\delta\delta\phi\rvert$	Overall average
	%*B*	*T* (°C)		
Group-1	27	52	0.005	
	28		0.005	
	29		0.006	
	30		0.006	
	31		0.007	0.006
Group-2	12	76	0.002	
	13		0.003	
	14		0.004	
	15		0.003	
	16		0.002	
	18		0.002	
	19		0.003	
	20		0.003	0.003
Group-3	8	64	0.004	
	9		0.005	
	10		0.006	
	11		0.007	
	12		0.007	
	13		0.010	0.006
			Overall	0.005

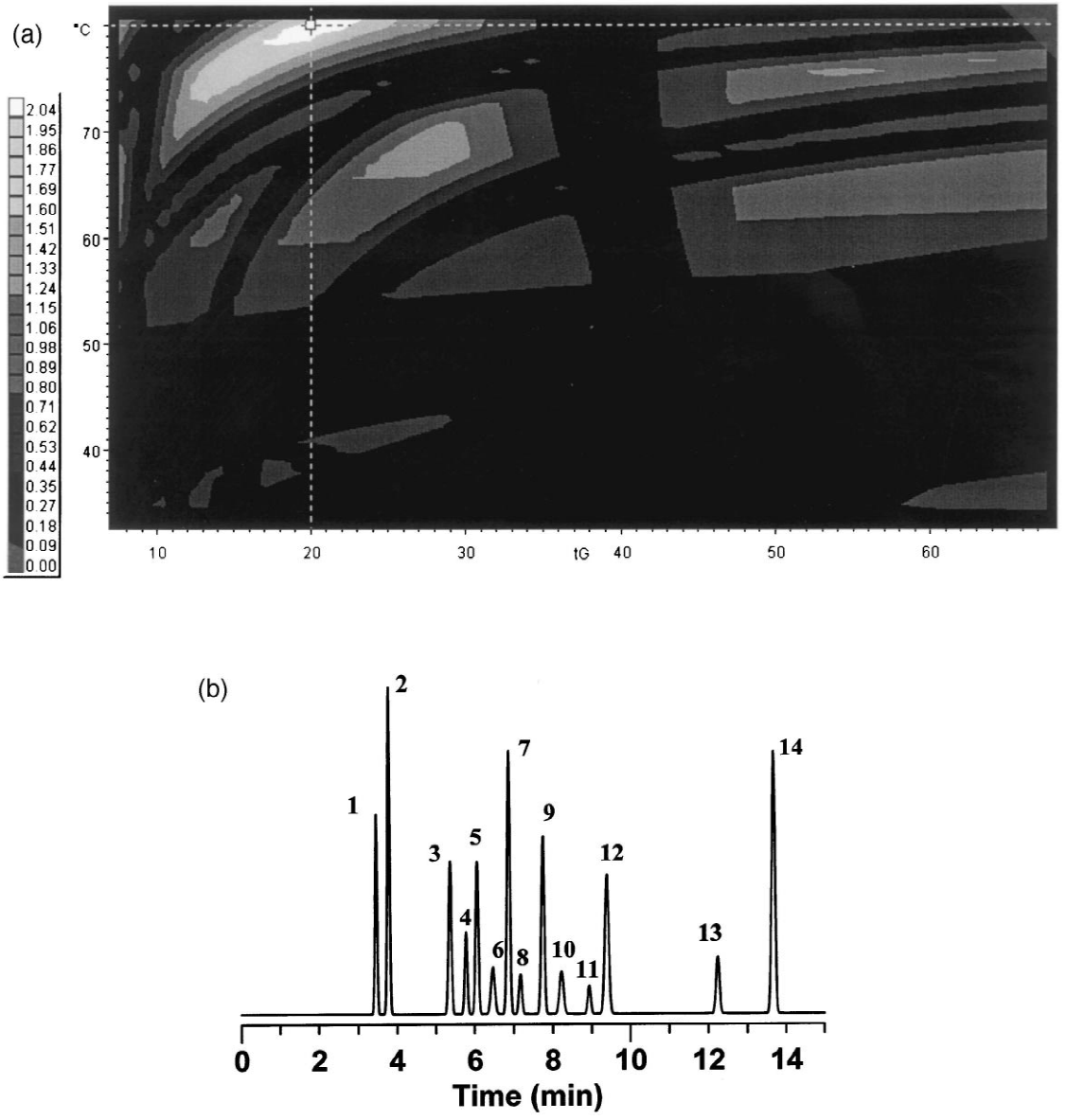


Fig. 8. Gradient separation of the 14-component mixture of substituted benzoic acids and anilines. Sample: (1) PA; (2) 2NB; (3) 35DMA; (4) 3CB; (5) 2FB; (6) 4ClA; (7) 3NB; (8) 2ClB; (9) 3FB; (10) 2ClA; (11) 26DMB; (12) 2ClA; (13) 34DClA; (14) 35DClA. Conditions as in the Experimental section, except for temperature and gradient time. (a) Resolution map; (b) optimized separation for $T=80$°C and $t_G=20$ min. Cursor in (a) marks conditions for maximum resolution.

separation of Fig. 6c with the experimental run and corrected conditions (Fig. 7b). Values of $|\delta\delta\phi|$ are seen to be negligible ($|\delta\delta\phi|=0.000$) for the separation of the critical bands 4ClA, 3CB and 2FB from each other. Resolution is predicted less well for early bands PA, 2NB and 35DMA (avg. $|\delta\delta\phi|=0.012$), but this is unimportant, because these bands are very well resolved ($R_s>5$). Note also the larger error in

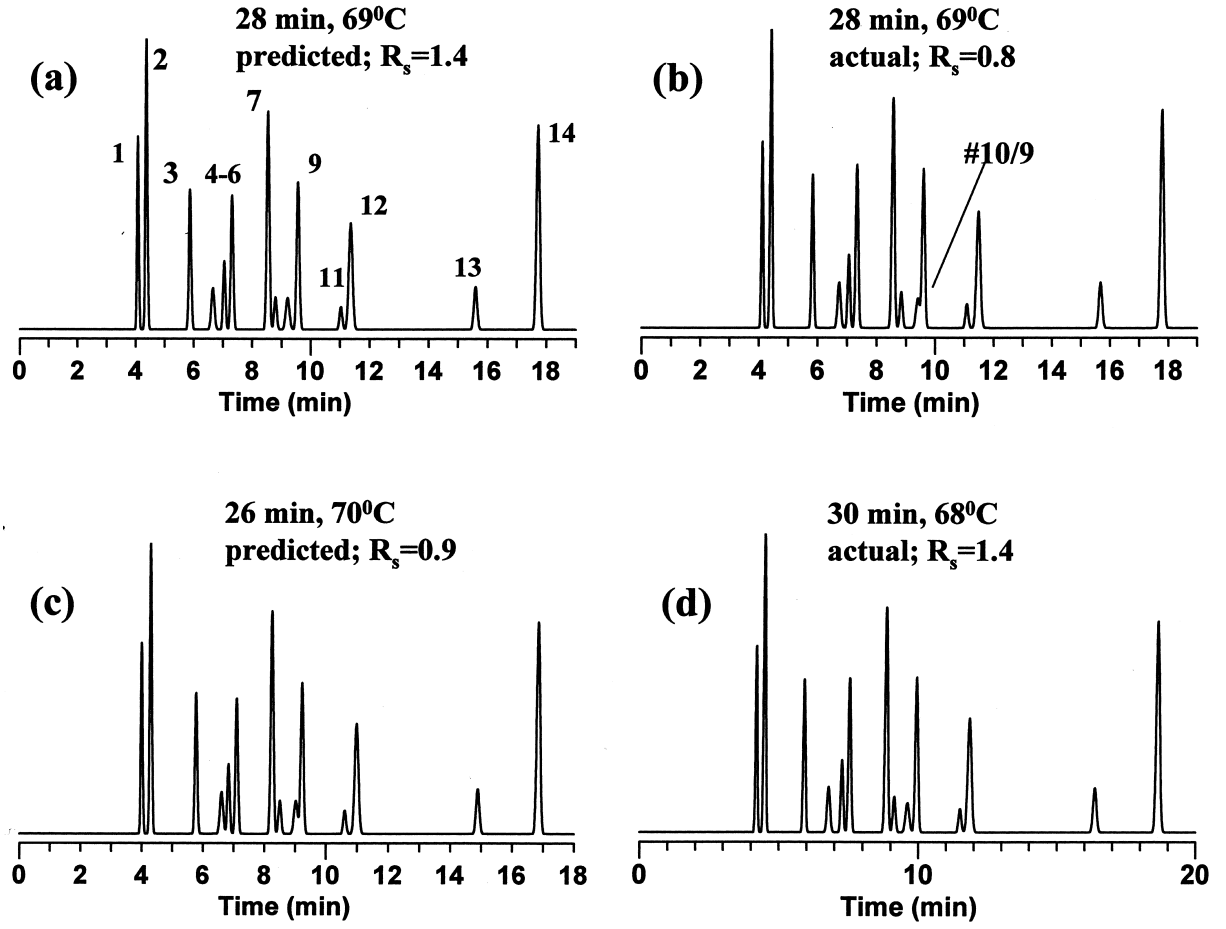


Fig. 9. Application of the ''reflection'' technique for correction of a predicted gradient separation. Sample: substituted anilines and benzoic acids, labeling as in Fig. 8. (a) Predicted (semi-optimized) separation for indicated conditions; (b) actual separation for conditions of (a); (c) best match of predicted separation to (b); (d) actual separation for ''reflected'' conditions. Other conditions described in the Experimental section. See text for details.

Tables 2 and 4 for smaller k values (see footnote b), corresponding to extrapolation outside the gradient range $4<k^*<12$; this is in agreement with previous studies [10].

4.3.2. Gradient predictions

Predictions of gradient separation from gradient input runs are less likely to be in error [10]. However, some samples exhibit a significant failure of Eq. (2) when T is varied, and in these cases significant computer-simulation errors can result when t_G and especially T are varied over wide limits. One such situation, which applies to the present experimental system, is any sample that contains partially-ionized acids or bases under the conditions of separation. Failure of Eq. (2) in this instance largely arises from change in solute pK_a values with temperature.

Fig. 8a shows the resolution map for the separation of the present aniline/benzoic acid sample as a function of T and t_G. Maximum resolution is observed for $T=80$°C and $t_G=20$ min ($R_s=2.1$); the predicted separation for these conditions is shown in Fig. 8b. Because the optimum temperature for the

Table 4
Comparison of corrected prediction of optimized separation of the group-1 sample (11% *B*, 64°C) with the experimental separation (10% *B*, 64°C)

Solute	Retention time (min)		Error $\delta\phi$[a]	Error $\lvert\delta\delta\phi\rvert$[a]	k[b]
	Predicted	Actual			
PA	2.32	2.84	0.027	0.015	1.7
2NB	3.06	3.49	0.015	0.009	2.3
35DMA	4.29	4.56	0.006	0.001	3.4
4CLA	5.85	6.32	0.007	0.000	5.1
3CB	6.32	6.86	0.008	0.000	5.6
2FB	6.77	7.38	0.008	–	6.1
average				0.007	

[a] Values calculated as described in [10].
[b] Isocratic predictions for $4<k<12$ are interpolations and therefore more reliable; the average error $\lvert\delta\delta\phi\rvert$for interpolations is ±0.000, versus ±0.008 for extrapolations ($k<4$).

separation of Fig. 8b (80°C) is close to a value used for the input runs (77°C), the actual separation is expected to agree closely with that predicted in Fig. 8b. Therefore, correction of this prediction (Fig. 8b) by the ''reflection'' technique is unlikely to be required. A secondary optimum is observed in Fig. 8a for $T=69$°C and $t_G=28$ min, and these conditions are more different from the input conditions (38 and 77°C, 13 and 39 min). Larger errors in the predicted separation for these conditions were therefore anticipated, making this example a possible candidate for the ''reflection'' technique. Fig. 9a shows the predicted separation for $T=69$°C and $t_G=28$ min, with $R_s=1.4$. The actual separation for these conditions is shown in Fig. 9b, where the resulting resolution is seen to be disappointing: $R_s=0.8$ (bands #9 and 10, which elute between 9 and 10 min, overlap). Several separations with T equal to 67–71°C and t_G equal 25–31 min were predicted, in order to obtain the best match to the actual separation of Fig. 9b: $T=70$°C and $t_G=26$ min (equivalent to ''errors'' of $+1$°C and -2 min). The latter predicted separation is shown in Fig. 9c and is seen to agree well with that of Fig. 9b. ''Reflection'' of these latter conditions relative to those of Fig. 9a suggests that an actual separation similar to that of Fig. 9a will be found for $T=69-1=68$°C and $t_G=28+2=30$ min; the latter separation is shown in Fig. 9d. As expected, this separation is indeed closely similar to that of Fig. 9a, with the same resolution ($R_s=1.4$).

5. Conclusions

For a wide range in sample types, previously reported studies have shown that the simultaneous optimization of temperature T and gradient time t_G is a powerful means for optimizing selectivity and maximizing sample resolution in gradient elution. For samples that can be separated isocratically, theory informs us that the simultaneous optimization of temperature and solvent strength (%*B*) should likewise be advantageous. Previously described software for the optimization of T and t_G (gradient elution) has now been expanded (DryLab 2000 for Windows) for the more convenient optimization of T and %*B* (isocratic separation). While either isocratic or gradient experiments can be used as input for the prediction of isocratic separation, the use of gradient input data is preferred. In the present study, both the advantages and limitations of this approach were demonstrated for the separation of various mixtures of substituted anilines and benzoic acids.

Predictions based on computer simulation can be in error for various reasons, and errors in the prediction of isocratic separation from initial gradient experiments are sometimes large enough to be unacceptable. This will be apparent whenever an experimental (confirmatory) run is compared with a predicted separation for preferred (''optimized'') conditions. When the experimental separation proves to be disappointing, a small adjustment in experimental conditions (T and/or t_G) usually will result in a separation similar to that first predicted as optimum. These adjusted conditions can be determined conveniently by computer simulation via the use of a so-called ''reflection'' procedure. This approach has been illustrated in the present study for the more accurate prediction of both isocratic and gradient separations based on initial gradient experiments.

If isocratic experiments are used as inputs for computer predictions of isocratic separation as a function of T and t_G, it is necessary to estimate appropriate values of %*B* for the input experiments. This in turn requires a knowledge of the temperature dependence of retention: $\%/°C \equiv -dk/dT$. It was found that neutral sample molecules have values of $\%/°C = 1–2\%/°C$, while ionizable molecules exhibit

more variable values of %/°C; e.g., 0–2%/°C in most cases, with occasional negative values of %/°C being observed (i.e., increase in t_G with increase in T). It appears that %/°C values usually decrease as the fractional ionization of an acid or base solute increases.

6. Glossary of terms

A, B	Constants in Eq. (2)
b	Gradient steepness parameter equal to $V_m \Delta\phi S/(t_G F)$
B	Refers to solvent B (''B-solvent'') in RP-LC
F	Flow-rate (ml/min)
k	Retention factor
k*	Gradient retention factor [Eq. (3)]
k_o	Value of k for a solute at the beginning of gradient elution
k_w	Retention factor for water as mobile phase (extrapolated value)
k_1, k_2	Value of k at $T=T_1$ and T_2, respectively
RP-LC	Reversed-phase liquid chromatography
S	$d(\log k)/d\phi$ [Eq. (1)]
t_o	Column dead-time
t_D	Equipment dwell time, equal to V_D/F
t_G	Gradient time (min)
t_R	Retention time (min)
T	Temperature (°C)
T_K	Temperature (°K)
T_1, T_2	Two different temperatures T
V_m	Column dead volume (ml)
α	separation factor, equal to ratio of k-values for two adjacent bands
ϕ	Volume fraction of B-solvent
$\delta\phi$	Error in predicted retention time expressed as equivalent change in ϕ
$\lvert\delta\delta\phi\rvert$	Absolute error in predicted retention-time difference (and corresponding resolution) for two adjacent bands (see further discussion of [10])
$\Delta\phi$	Change in ϕ from beginning to end of gradient
%/°C	Temperature coefficient of retention; equal to dk/dT

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Appendix A. Retention in RP-LC as a function of temperature

An average value of the change in k with T ($-dk/dT \equiv$ %/°C) can be determined from retention values k_1 and k_2 measured at temperatures T_1 and T_2:

$$\log(1 + 0.01[\%/°C]) = -\log(k_2/k_1)/(T_2 - T_1) \quad (A.1)$$

This change in k as a result of change in T can be reversed or compensated by a corresponding change in %B ($\delta\phi$), which can be calculated from Eqs. (1) and (A.1) (assuming an average value of $S \approx 4$ ([15], and no change in S with T [16]):

$$\delta\phi = (1/4)\{\log(1 + 0.01[\%/°C])\}(T_1 - T_2) \quad (A.2)$$

Values of %/°C are normally positive, corresponding to negative retention enthalpies, so for $T_2 > T_1$, %B or ϕ must be decreased to compensate for reduced retention at the higher temperature. This requires an estimate of the value of %/°C. A widely used rule for RP-LC retention as a function of temperature is that a 1°C increase in T will reduce k by 1 to 2% (p. 242 of [13]); i.e., %/°C = 1–2. However, no systematic verification of this relationship has been reported for solutes of varied structure and for a range in separation conditions (e.g., %B, pH, etc. varying).

Neutral solutes

Fig. 10a summarizes values of %/°C as a function of k, for the RP-LC separation of a 5-component mixture of dialkylphthalates with acetonitrile (ACN) as B-solvent. This plot is derived from data reported in [17], and it is seen that these values of %/°C are relatively independent of the value of %B. Fig. 10b

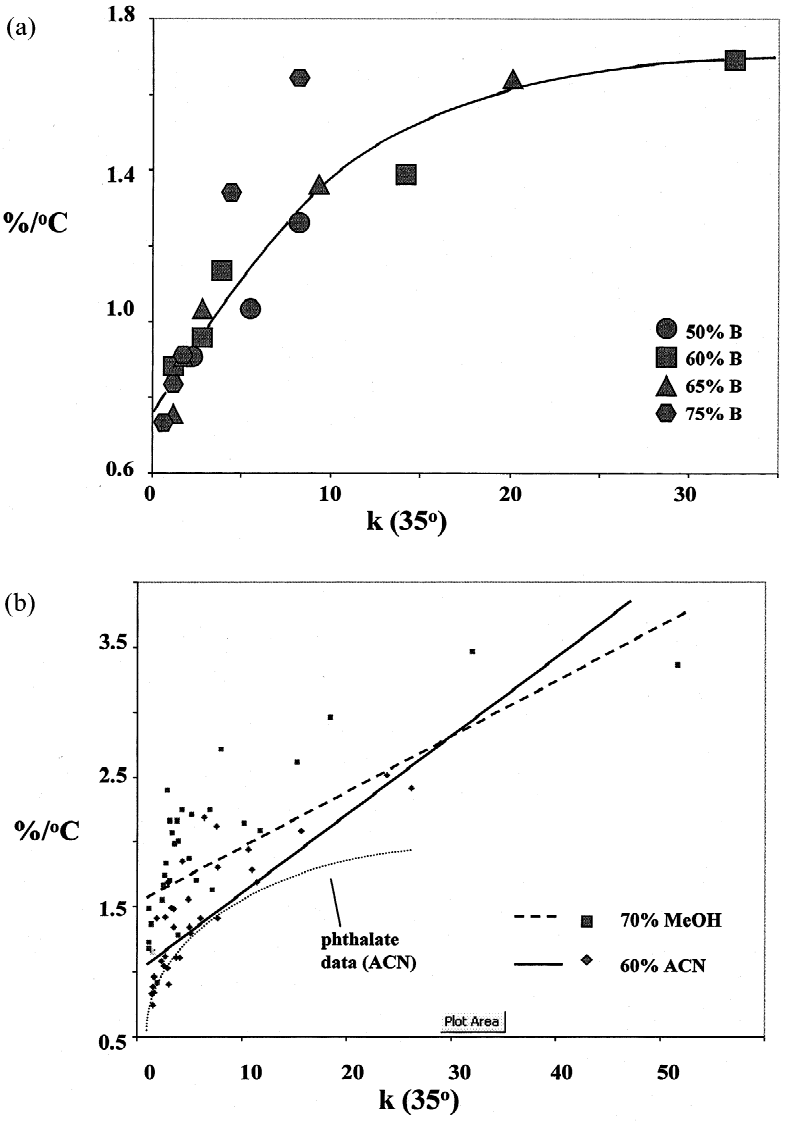


Fig. 10. Dependence of solute retention on temperature T for neutral compounds as a function of solute k-value at 35°C. (%/°C) is the average %-decrease in t_G for a 1°C increase in T over the temperature range studied. (a) Dialkylphthalate sample for different values of %B, from data of [17]; (b) nitro-aromatic sample for different B-solvents (methanol and acetonitrile), from data of [18]. Values of (%/°C) calculated from Eq. (2). Note that experimental error is believed to be greater for the data of Fig. 10b versus the data of 10a (A. Robbat, Jr., private communication).

shows a similar plot for 34 nitro-substituted aromatic compounds from the data of [18], for both ACN and methanol (MeOH) as B-solvents. Data for the dialkylphthalates of Fig. 10a are indicated by the lower dotted curve. The various straight-line curves of Fig. 10b are in each case least-square fits. The data of Fig. 10b suggest slightly greater values of %/°C for MeOH versus ACN, especially at lower values of k, but the difference is within the scatter of individual data points around the best-fit curves. Overall, the data of Fig. 10b suggest that average values of %/°C range from about 1.0 for $k=1$ to 2.0 for $k=20$; i.e., in good agreement with the above rule-of-thumb (1–2% decrease in k for each 1°C

increase in T). An average value of %/°C = 1.5 in combination with Eq. (A.2) allows estimates of values of %B for the higher temperature runs of Fig. 2a, which will avoid crowding of early peaks as in Fig. 2f. Thus, for the example of Fig. 2a, $T_2 - T_1 =$ 40°C, so from Eq. (A.2), $\delta\phi = (1/4)\ \log(1.015) \times 40 = 0.065$. This suggests that the mobile phase for the experimental runs at 75°C should be 6.5% B lower than at 35°C; i.e., if 27 and 42% B are used at 35°C, the corresponding values at 75°C should be 20.5 and 35.5% B. The revised choices for isocratic experiments at 75°C, versus those selected in Figs.

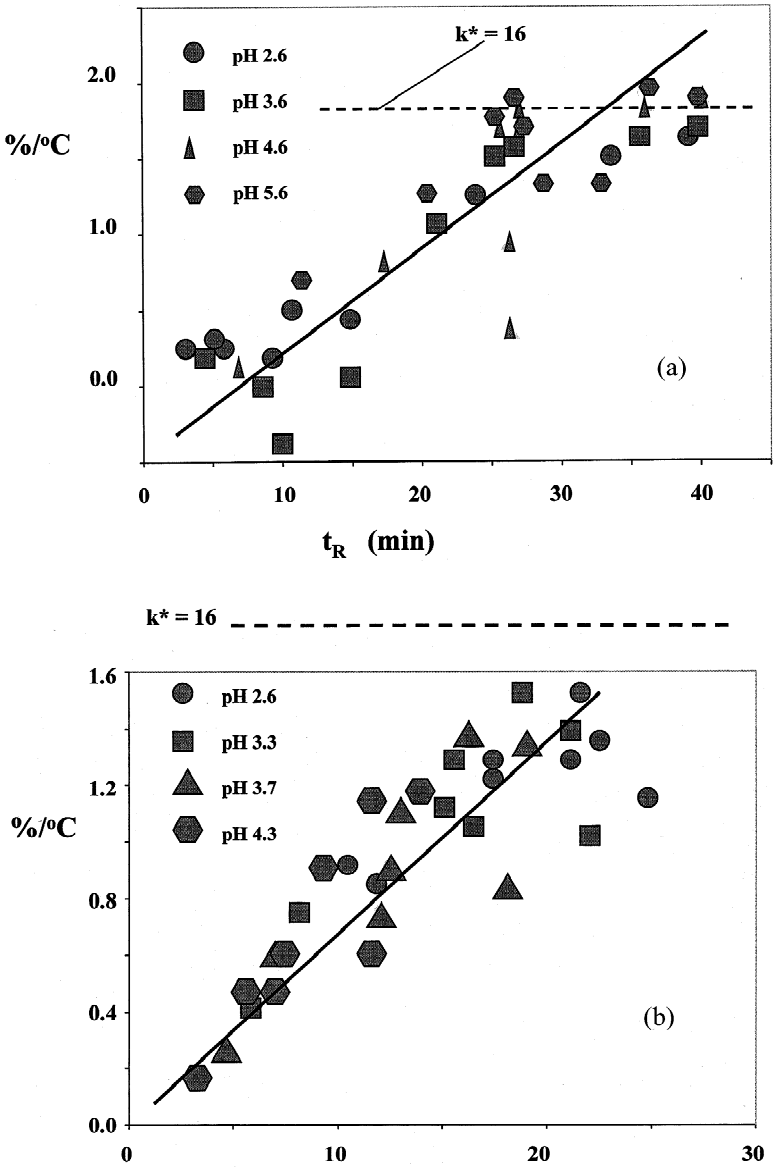


Fig. 11. Dependence of solute retention on temperature T for ionic compounds as a function of solute k-value at 35°C. (%/°C) is the average %-decrease in k for a 1°C increase in T. (a) Substituted benzoic acid sample for different values of mobile phase pH, from data of [9]; (b) substituted aniline sample for different values of mobile phase pH, from data of [9]. Values of (%/°C) calculated from Eq. (A.6).

2e and f are shown in Fig. 2g and h. The undesirable crowding of early bands in Fig. 2f is seen to be alleviated somewhat in Fig. 2h.

Ionic solutes

A previous study [1] has described gradient elution experiments for samples composed of substituted anilines or benzoic acids, where both T and pH were varied. In gradient elution, retention t_R can be approximated [13] by

$$t_R = (t_o/b)\log(2.3k_o b) + t_o + t_D \quad (A.3)$$

The change in t_R as a result of change in T can then be estimated by

$$\delta t_R = (t_G/\Delta\phi S)\log(k_2/k_1) \quad (A.4)$$

The quantity $\Delta\phi$ refers to the change in ϕ during the gradient, and k_1 and k_2 refer to values of k at the start of the gradient (k_o) at temperatures T_1 and T_2; see Glossary of Terms for other symbols in Eq. (A.3). Eq. (A.4) assumes that S in gradient elution does not change with T, and this has been found to be a good approximation [16]. For this reason, (k_2/k_1) in Eq. (A.4) can be identified with the same quantity in Eq. (A.1). This in turn leads with Eq. (A.1) to Eq. (A.5)

$$\log(1 + 0.01[\%/°C]) = \delta t_R\,\Delta\phi S/(t_G[T_2 - T_1]) \quad (A.5)$$

or with $S \approx 4$,

$$\log(1 + 0.01[\%/°C]) = 4\delta t_R\,\Delta\phi/(t_G[T_2 - T_1]) \quad (A.6)$$

Values of %/°C calculated by means of Eq. (A.6) from the data of [1] are summarized for substituted anilines in Fig. 11a and substituted benzoic acids in Fig. 11b. Because these data are derived from linear-gradient RP-LC experiments, the effective retention factor k^* in these experiments is approximately constant for all solutes ($k^* \approx 16$, unlike the case for isocratic elution). From Fig. 11b with $k = k^* = 16$, we estimate that %/°C should equal about 1.8 for all solutes (ACN is B-solvent), as indicated by the dashed line in Figs. 11a and b. For the separations summarized in Figs. 11a and b, it is seen that values of %/°C cluster around the expected value of 1.8 when $t_R > 25$ min, but %/°C approaches zero as t_R becomes small. There are also instances [9] of *negative* values of %/°C for ionized compounds, corresponding to an *increase* in retention at higher temperatures.

Several explanations for the dependence of %/°C on t_R in Fig. 11 are possible. First, values of k^* are constant for solutes that are well retained at the start of gradient elution, but k^* tends to be smaller for bands that elute early in the gradient (see Eqs. (19) and (20) of [15]). Second, smaller values of t_R in Fig. 11 correspond to more highly ionized solutes, and it is possible that ionized solutes have lower values of %/°C. Finally, the relative ionization of partially ionized solutes will change with T, if the value of pK_a for the solute is temperature dependent. We believe that the latter explanation is most likely, in which case it follows from Fig. 11 that solute ionization must generally decrease at higher temperatures. Decreased ionization would mean increased retention, which would then offset the normal decrease of retention with increasing temperature. Regardless of an explanation for the data of Fig. 10, *these data suggest some uncertainty in any estimation of changes in %B that can offset the effects of changing temperature on sample retention* (as in Fig. 2), especially for acidic or basic samples.

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