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Combined use of temperature and solvent strength in reversed-phase gradient elution
I. Predicting separation as a function of temperature and gradient conditions

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Abstract

It has been shown previously that computer simulation based on two initial experiments can predict separation in reversed-phase gradient elution as a function of gradient conditions (gradient steepness, gradient range and gradient shape) and column conditions (column length, flow-rate and particle size). The present study extends this capability for changes in temperature. Four initial experiments (two different gradient times, two different temperatures) provide input data that allow predictions of separation as a function of temperature as well as gradient and column conditions. A semi-empirical relationship, $t_R = a + bT$, is able to relate gradient retention time t_R to column temperature T (other conditions constant). The accuracy of this approach has been evaluated for 102 solutes and a variety of experimental conditions, including the use of five different HPLC instruments (four different models).

Keywords: Gradient elution; Solvent strength; Column temperature; Retention prediction; Computer simulation

1. Introduction

Several systematic approaches for reversed-phase HPLC (RP-LC) method development have been described [1–4]. In most cases, an attempt is made to optimize sample retention (values of the retention factor, k), column efficiency (plate number, N) and selectivity (separation factors, α). Major emphasis is usually given to the optimization of selectivity, often using a preselected series of experiments plus a computer program for predicting retention (k and α) as a function of one or more experimental variables. In the past, favored method development strategies have been based on varying experimental conditions

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that are believed to have the largest effect on α; e.g. solvent type, solvent strength (%B) and column type for neutral samples, or pH and ion-pair-reagent concentration for ionic samples.

The choice of a variable parameter (%B, pH, etc.) for optimizing RP-LC selectivity should be guided by additional considerations, aside from the desirability of large changes in α. The high-efficiency columns now in use for RP-LC permit the baseline separation of previously unresolved bands after only a small change in α. Therefore, maximum changes in α are rarely required. Furthermore, several "practical" factors are important in the development of routine RP-LC assays [5,6]: experimental convenience, method ruggedness and the easy applicability of computer simulation as part of method development. If computer simulation is used, peak tracking should be feasible and the number of required experiments should be as small as possible. Table 1 summarizes some advantages (+, ++) and disadvantages (−, −−) when changing different variables for the purpose of controlling selectivity; variables 4–9 and the use of THF as solvent have definite disadvantages in both method development and final routine assays. The use of solvent strength (isocratic %B or gradient steepness b), temperature and methanol and/or acetonitrile as solvents, on the other hand, is not limited in this way. This suggests that changes in temperature as a means of controlling selectivity may be worthwhile in HPLC method development, either alone or in combination with other "favored" variables.

In the present study, a new approach to RP-LC method development is under investigation: the use of gradient elution with temperature and gradient steepness b as variable parameters for the optimization of selectivity and separation. Although this procedure is easily adaptable to isocratic separation, its use in a gradient mode is both more convenient and more powerful as a means of adjusting selectivity. The use of gradient elution with temperature and solvent strength as variable parameters is free from several "practical" problems associated with other experimental variables (Table 1 and discussion of Refs. [5,6]). This approach is also convenient to carry out. Once the A- and B-solvents have been formulated and a column selected, further changes in conditions (as part of method development) can be effected without preparing new mobile phases or changing the column. In one report [7,8], where a simplified version of this method development approach was described for several peptide and protein samples, large changes in selectivity resulted from changes in temperature and gradient steepness. For

Table 1
Some factors that affect the choice of variables for HPLC method development (++, very favorable; +, favorable; −, unfavorable; −−, very unfavorable)

Variable	Impact on different factors						
	A	B	C	D	E	F	G
1. Solvent strength (%B)	+	++	++	+	+	++	+
2. Temperature	?	++	++	+	+	++	+
3. Solvent type							
ACN	+		+	+	+		+
MeOH	+		+	+			+
THF	++	−	−	−	−	−	
4. pH	++	−	+	−−	−	−	−−
5. Ion-pairing	++	−	−−	−−	−		
6. Column type	+	−	−		+	−	−
7. Column source	−	−	−	−−	+	−	−
8. Mobile phase additives			−	−−	−		
9. Buffer type and concentration	−				−	−	

See also Refs. [5,6].
A, ability to change α; B, experimental convenience, need for operator intervention; C, column equilibration (fast or slow); D, method ruggedness; E, compatible with low-UV detection; F, large or small number of runs required to select optimum value of variable; G, peak tracking problems.

isocratic separation, a change in %B is equivalent to a change in gradient steepness b [9].

Several goals were visualized for the present study. First, accurate mathematical relationships were required that would allow the prediction of separation (retention times, bandwidths and resolution) as a function of any temperature or gradient steepness, as well as for further changes in gradient conditions (initial and final %B, gradient shape) or column conditions (column length, particle size, flow-rate). This goal is addressed in the present paper, which describes and evaluates a procedure for the accurate prediction of retention and resolution from four initial runs.

Second, selectivity measurements as a function of temperature, gradient steepness and a variety of solute types were needed for the evaluation of temperature plus gradient steepness as variable parameters for controlling selectivity. This would address an important question: is the combined use of these two variables an effective method development approach? And is this approach more effective for some sample types than for others? Part II of this series [10] develops a theoretical model that allows selectivity as a function of temperature and gradient steepness to be compared for different samples. Parts III [11] and IV [12] apply this theory to samples of different kinds, in order to answer the preceding questions.

Finally, the dependence of temperature and gradient-steepness selectivity on initial experimental conditions (e.g. pH, column type, etc.) was of interest, as a guide for selecting optimal starting conditions for a given sample. Parts III [11] and IV [12] present pertinent data for a wide range of samples.

2. Theory

2.1. Prediction of gradient retention as a function of gradient conditions and temperature

If isocratic retention can be predicted as a function of temperature and solvent strength (%B), the basic theory of gradient elution [13,14] allows a similar prediction of retention in gradient separations. Melander et al. [15] have reported theory-based correlations of isocratic retention times with temperature and %B. However, the reported accuracy of these predictions was ±6–8%, which may not be adequate for method development purposes. We therefore adopted a more empirical approach.

2.1.1. Retention vs. %B and gradient steepness

When solvent strength (%B) is varied in isocratic RP-LC (other conditions constant), solute retention is related [11] to the volume fraction of the B-solvent ϕ (=0.01%B) as

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

where k_w is the (extrapolated) value of k for $\phi = 0$ (water as mobile phase) and S is a constant for each solute. In gradient elution [9], retention time t_R is given as

$$t_R = (t_o/b)\log(2.3k_o b + 1) + t_o + t_D \quad (2)$$

where k_o is the value of k at the start of the gradient; k_o is related via Eq. (1) to the value of ϕ at the start of the gradient (ϕ_o)

$$\log k_o = \log k_w - S\phi_o \quad (3)$$

The gradient-steepness parameter b is given by

$$b = V_m \Delta\phi\, S/(t_G F) \quad (4)$$

V_m is the column dead-volume (ml), $\Delta\phi$ is the change in ϕ from the start to the end of the gradient, t_G is gradient time (min) and F is flow-rate (ml/min). The quantities t_o and t_D (Eq. (2)) are the column dead-time and gradient dwell time, respectively.

Values of S and k_w for each solute can be determined from two gradient runs where only gradient time t_G is varied [9]. Given values of S and k_w for each solute in a sample, retention times can be calculated for other gradient conditions: change in t_G, F, column dimensions, initial (ϕ_o) and final (ϕ_f) values of ϕ, and gradient shape. The accuracy of this approach, using computer simulation, has been confirmed in several studies [16–22]. If values of k_w and S can be determined as a function of temperature, then retention can be predicted accurately for all gradient conditions and any temperature. Previous studies [8,23,24] and especially work summarized in Part II [11] suggest that S does not vary much with temperature.

2.1.2. Retention vs. temperature

With few exceptions, isocratic retention as a function of temperature has been reported to be described by the van't Hoff relationship [25]

$$\log k = A + B/T \tag{5}$$

A and B are constants for a given compound and set of experimental conditions and T is the absolute temperature. The coefficient A is a function of the standard state entropy of retention and the phase ratio, while B is proportional to the standard state enthalpy of retention. By extension of Eq. (5), we can write

$$\log k_o = A + B/T \tag{6}$$

For well retained peaks ($k_o \gg 1$), Eq. (2) reduces to

$$t_R = (t_o/b)\log(2.3\, k_o b) + t_o + t_D \tag{7}$$

If S does not vary with temperature, values of b can be assumed constant for a change in temperature (Eq. (4), other conditions constant). A change in temperature from T_a to T_b will then cause a change in t_R equal to (Eq. (7))

$$(t_R)_b - (t_R)_a = \Delta t_R = (t_o/b)\log(k_{ob}/k_{oa}) \tag{7a}$$

and from Eq. (6)

$$\Delta t_R = (t_o/b)B([1/T_a] - [1/T_b]) \tag{8}$$

or

$$B = (b/t_o)\,\Delta t_R/([1/T_a] - [1/T_b]) \tag{8a}$$

2.2. Prediction of bandwidth as a function of gradient conditions and temperature

A well developed theory exists for bandwidth in gradient elution as a function of experimental conditions [9]. The baseline bandwidth W is given as

$$W = (4/N^{1/2})(GJ)t_o(1 + 0.42b) \tag{9}$$

where N is the column plate number, G is a gradient compression factor and J is a poorly-understood correction term [26]. For a wide range in values of b, $(GJ) \approx 1.1$. N is a function of temperature [27], and, in theory, N should increase as temperature increases. Reported experimental studies show that this is often the case, but exceptions have been noted. If initial experiments are carried out at two different temperatures, values of N can be calculated at each temperature from Eq. (9). Values of N at other temperatures can then be estimated by linear interpolation or extrapolation:

$$N \approx a' + b'T \tag{10}$$

where a' and b' are constants for a given solute as T is varied (other conditions constant).

3. Experimental

Data reported here (Part I) were obtained in five different laboratories. Tables 2–6 summarize the solutes studied in laboratories A–E. Resulting data sets for different groups of compounds will be referred to by the laboratory designation (A–E). Experimental conditions varied with the laboratory and are described below. Different compounds in the various experiments included neutral, anionic (acidic) and cationic (basic) species. Mobile phases were in each case prepared from HPLC-grade materials.

Table 2
Solutes used in studies carried out in laboratory A (benzoic acids and anilines)

Substituted benzoic acids	
1	Phthalic acid
2	2-Nitrobenzoic acid
3	3-Cyanobenzoic acid
4	2-Fluorobenzoic acid
5	2-Chlorobenzoic acid
6	3-Nitrobenzoic acid
7	3-Fluorobenzoic acid
8	2,6-Dimethylbenzoic acid
Substituted anilines	
9	4-Methoxyaniline
10	3-Methylaniline
11	*N*-Ethylaniline
12	3,5-Dimethylaniline
13	4-Chloroaniline
14	3-Chloroaniline
15	2-Chloroaniline
16	3,4-Dichloroaniline
17	3,5-Dichloroaniline

Table 3
Solutes used in studies carried out in laboratory B (nitroalkanes and miscellaneous drugs)

Substituted 1-nitro-alkanes (internal standards)	
1	Nitromethane
2	Nitroethane
3	1-Nitropropane
4	1-Nitrobutane
5	1-Nitropentane
6	1-Nitrohexane
7	1-Nitroheptane
8	1-Nitrooctane
9	1-Nitrononane
10	1-Nitrodecane

Miscellaneous drugs

Bases		*Acids and neutrals*	
1	Nicotine	1	Acetaminophen
2	*m*-Aminobenzamide	2	β-Hydroxytheophylline
3	Sulfanilamide	3	Pyrithydione
4	α-Methylamino(Me) benzyl alcohol	4	Aprobarbital
5	Phenylpropanolamine	5	Doxapram
6	Morphine	6	Indole-3-carboxaldehyde
7	5-Hydroxyquinoline	7	Salicyclic acid
8	Tranylcypromine	8	Butabarbital
9	Amphetamine	9	Butethal
10	Tripelennamine	10	Acetophenone
11	Methamphetamine	11	Cortisone
12	Codeine	12	Oxazepam
13	*N*-Acetylprocainamide	13	2-Naphthoxyacetic acid
14	Phentermine	14	Chlorpromazine
15	Ethylmorphine	15	Fluoxymesterone
16	Sulfmethanzine	16	Flunitrazepam
17	Brucine	17	Lormetazepam
18	Clenbuterol	18	Chloroxylenol
19	Chlorodiazepoxide	19	Butylparaben
20	Vincamine	20	Diflunisal
21	Desipramine	21	Danthron
22	Imipramine	22	Phenylbutazone
		23	Mefenamic acid
		24	Biphenyl
		25	Danazol

Table 4
Solutes used in studies carried out in laboratory C ((proprietary) herbicide impurities)

1	$C_6H_9N_3O_2$
2	$C_7H_{10}N_2O_4S_2$
3	$C_7H_{10}N_4O_3$
4	$C_{12}H_15N_3O_2S$
5	$C_{14}H_{14}N_2O_3$
6	$C_{13}H_{13}N_3O_4$
7	$C_{13}H_{16}N_6O_5$
8	$C_{14}H_{16}BrN_5O_7S_2$
9	$C_{20}H_{20}N_6O_8S_2$

3.1. Laboratory A (Zhu et al.)

3.1.1. Equipment

A Beckman System Gold HPLC system (Fullerton, CA) was used with a contact-type column heater (CH-105, Eldex Laboratories, San Carlos, CA). The temperature of the column heater was measured as a function of the temperature setting and all temperatures reported are measured values. The injection valve and incoming mobile phase were thermostated

Table 5
Solutes used in studies carried out in laboratory D (pharmaceuticals and intermediates)

1	3,4-Dihydro-6-methoxynapthalen-2(1H)-one ($C_{11}H_{12}O_2$)
2	4-Phenyl-1,3-cyclohexanedione ($C_{12}H_{12}O_2$)
3	2-Indanone (C_9H_8O)
4	4-Chloroquinazoline ($C_8H_5N_2Cl$)
5	γ-Acetylbenzenebutanoic acid, methyl ester ($C_{13}H_{16}O_3$)
6	4,4a,9,10-Tetrahydro-7-methoxy-4a-methyl, 2(3H)-phenanthrenone ($C_{16}H_{20}O_2$)
7	1-Benzoyl-1H-indole ($C_{15}H_{11}NO$)
8	1-(Phenylmethyl)-1H-indole ($C_{15}H_{13}N$)
9	*N,N*-Dibenzyl-3-phenyl-alanine benzyl ester ($C_{30}H_{25}NO_2$)

at the column temperature. The system dwell volume was 1.9 ml.

3.1.2. Procedures

A 15×0.46 cm, 5 μm Zorbax SB-C18 column (Rockland Technologies, Newport, DE) was used for all experiments, with a flow-rate of 1.0 ml/min. The A-solvent consisted of an aqueous mixture of 25 m*M* citric acid adjusted with dibasic sodium phosphate to the desired pH value (pH=2.6, 3.2, 3.7 and 4.3 for benzoic acid sample; pH=2.6, 3.6, 4.6 and 5.6 for aniline sample). The B-solvent was acetonitrile. Gradients were started at 5% B and were run at either 1%/min or 3%/min. Values of pH were determined for the A-solvent at ambient temperature.

3.2. Laboratory B (Hill)

3.2.1. Equipment and procedures

These are described in Ref. [28]; an HP 1090 HPLC system (Hewlett-Packard) was used with a 25×0.46 cm, 5 μm Zorbax Rx-C18 column (Rockland Technologies). Solvent A was aqueous 0.15 *M* H_3PO_4–0.05 *M* triethylamine; solvent B was 80% acetonitrile/solvent A; all gradients were 0–100% B in 20 or 60 min, preceded by a 2-min isocratic hold at 0% B. The flow-rate was 2.0 ml/min. The mobile phase entering the column was preheated to column temperature for some experiments, and not for others. The system dwell volume was 1.0 ml.

3.3. Laboratory C (Waeghe)

3.3.1. Equipment

An HP 1090 HPLC system (Series II, Hewlett-Packard) was used with preheating of the mobile phase. The system dwell volume was 1.0 ml.

3.3.2. Procedures

A 7.5×0.46 cm, 3.5 μm Zorbax SB-C8 column (Rockland Technologies) was used for all experiments, with a flow-rate of 2.0 ml/min. The A-solvent consisted of an aqueous solution of phosphoric acid (pH 2.75); the B-solvent was acetonitrile. Gradients were run from 5–95% B in times of 10, 20 and 30 min.

3.4. Laboratory D (Djordjevic)

3.4.1. Equipment

The HPLC system was an HP1050 (Hewlett-Packard) with a dwell volume of 1.2 ml and preheating of the mobile phase.

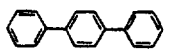
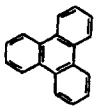
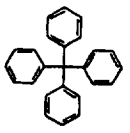
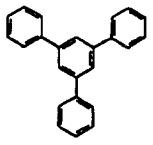
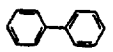
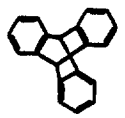
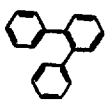
3.4.2. Procedures

A 25×0.40 cm, Nucleosil 120 C18 column (5 μm particles; Bischoff, Leonberg, Germany) was used for all experiments, with a flow-rate of 1.0 ml/min. The A-solvent consisted of 0.02% phosphoric acid in water; the B-solvent was acetonitrile. Gradients were run from 20–100% B in times of 30, 60 and 90 min.

3.5. Laboratory E (Sander)

Equipment and procedures have been described in Ref. [29]; the system dwell volume was 7.0 ml. Two different columns were used: (a) Hypersil Green PAH (Shandon, referred to as "polymeric") and (b) Zorbax-ODS (Rockland Technologies, referred to as "monomeric"), each in a 25×0.46 cm configuration. All separations were carried out with acetonitrile (ACN)/water gradients, at 1.0 ml/min. Columns were thermostated with a circulating fluid column jacket and a constant temperature bath. The fluid temperature was measured within the column jacket

Table 6
Test compounds used to evaluate shape selectivity by laboratory E (33)

Solute group	Solute	Structure	Structural characteristics
A	*p*-Terphenyl		Semi-planar, large L/B
	1,6-Diphenyl-hexatriene		
B	Pyrene		Planar, small L/B
	Triphenylene		
C	Tetraphenyl methane		Non-planar, bulky, small L/B
	1,3,5-Triphenylbenzene		
D	Biphenyl		Less retained
	Triptycene		
	o-Terphenyl		

with a thermocouple, and typically varied less than 0.2°C. The mobile phase was not preheated, nor was the temperature of the injection valve controlled.

Experimental retention times used to arrive at conclusions in this and the following three papers can be obtained either from one of the authors (LRS) or from the Journal of Chromatography.

3.6. Column temperature

Measured temperatures differed from those indicated by the temperature controller (set-points) for three of these HPLC systems (laboratories A–C). In each case, a linear relationship existed (±0.5°C) between measured and set-point temperatures. For

the contact heater used by laboratory A, the measured temperature T (°C) was related to the set-point temperatures by $T=0.4+0.939\ T'$. This resulted in a 5°C difference in temperatures when the set-point temperature was 75°C. Similar differences in measured and set-point temperatures were noted for the HP 1090 systems of laboratories B and C: $T=2.6+0.912\ T'$. Temperatures reported here are in every case measured values.

4. Results and discussion

4.1. Prediction of retention times in RP-LC gradient elution

Several different samples comprising 102 solutes (Tables 2–6) were used to investigate retention as a function of temperature and gradient steepness. For some of these samples, experimental conditions were varied further (change in pH). Because predictions of retention for varying gradient conditions (temperature constant) have been found to be generally reliable, the main question is whether the retention can be predicted accurately as temperature is varied, with other conditions held constant.

4.1.1. Experimental results vs. Eq. (8)

Fig. 1 tests the validity of Eq. (8) for several representative compounds (only temperature varying for a given compound). A straight line connects the two extreme data points for each solute in Fig. 1. These plots are approximately linear (as required by Eq. (8)), but there is a slight curvature of the plots for more retained compounds (Fig. 1a). This curvature could arise as a result of various assumptions in the derivation of Eq. (8): (a) a modest failure of Eq. (1) and/or Eq. (6), (b) non-constant S-values as temperature is varied (see discussion of Part II [10]), or (c) non-constant values of the coefficient B as %B

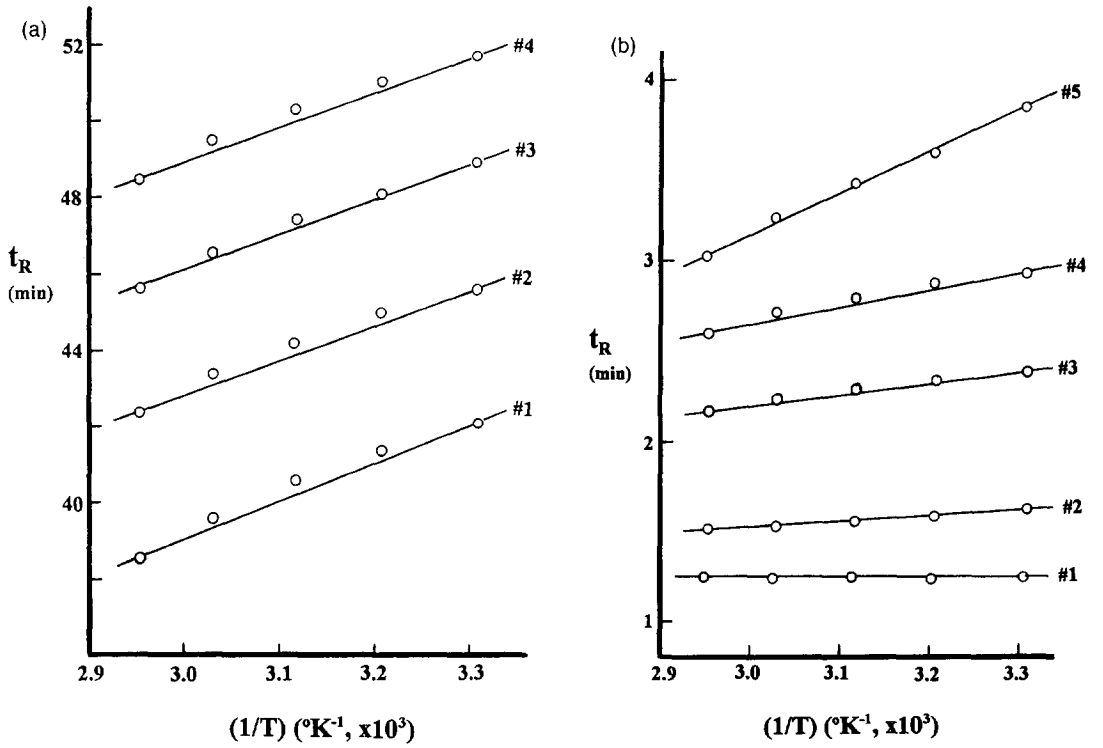


Fig. 1. Plots of retention time t_R vs. reciprocal temperature (K) for some compounds from Table 3. Gradient time is 60 min and other conditions are those of laboratory B (Section 3). (a) Compounds 1–4 are, respectively, nitroalkane solutes 7–10 of Table 2. (b) Compounds 1–5 are: 1, nicotine; 2, *m*-aminobenzamide; 3, nitromethane; 4, sulfanilamide; 5, α-methylamino(Me)benzyl alcohol.

is changed (effects [b] and [c], if significant, would be correlated). In addition, an incomplete thermostating of the mobile phase entering the column would be expected to lead to further deviations of experimental retention times from values predicted by Eq. (8).

The curvature of the plots of Fig. 1 was consistent for almost all data collected in this study. This will be illustrated by the data from laboratory B (nitroalkanes and drugs), some of which are shown in Fig. 1. Let the range in t_R values for each solute be normalized $(t_R)_n$, so that $(t_R)_n = 1$ for the lowest temperature (30.0°C) and 0 for the highest temperature (66.3°C). Values of $(t_R)_n$ were then calculated from retention data for the compounds of Table 3. These results were averaged for each intermediate temperature (39.5, 48.5 and 57.7°C) and gradient time (20 or 60 min) and are summarized in Table 7.

Several conclusions can be drawn from the data of Table 7 and predictions based on either of two a priori relationships:

$$t_R = a' + b'(1/T) \quad (11)$$

(another form of Eq. (8)) or

$$t_R = a' + b'T \quad (11a)$$

Table 7
Normalized retention times $(t_R)_n$ for solutes of Table 2 (average values)

Data set	Average value of $(t_R)_n$		
	39.5°C	48.5°C	57.7°C
Solutes 3–10 (nitroalkanes)			
$t_G = 20$ min	0.78	0.54	0.27
$t_G = 60$ min	0.79	0.56	0.29
Solutes 11–59 (drugs)			
$t_G = 20$ min	0.78	0.53	0.25
$t_G = 60$ min	0.78	0.50	0.29
Average[a] f(T)	0.78	0.53	0.27
Calculated[b]			
$1/T$	0.72	0.46	0.21
T	0.74	0.50	0.25

No pre-heating of the mobile phase; see Section 3 for other details.

[a] Average of experimental values of $(t_R)_n$; see Section 4.1.1.

[b] Values of $(t_R)_n$ predicted by either Eq. (11) ($1/T$) or Eq. (11a) (T).

(an empirical relationship) where a' and b' are constants for a given compound, only temperature varying. First, the experimental data show similar values of $(t_R)_n$ at a given temperature, for different compounds and different gradient times. That is, a single relationship (f[T] in Table 7) can be used to estimate $(t_R)_n$ as a function of temperature, which in turn would allow values of t_R to be estimated as a function of temperature. Second, f(T) differs from the functional dependence of t_R on $1/T$ predicted by Eq. (8) or Eq. (11); see "calculated $1/T$" in Table 7. At the approximate midpoint (48.5°C) of these $t_R - (1/T)$ plots as in Fig. 1, Eq. (11) is significantly in error (by an average of 0.07 units). Finally, there is better agreement with Eq. (11a) with an empirical, linear $t_R - T$ relationship; the average error at the midpoint is only 0.03 units. A fitting equation with more terms than Eq. (11a) (e.g. a quadratic) could be used to further improve the prediction of retention as a function of temperature. However, the following discussion will show that this is in most cases unnecessary.

4.1.2. Predicting retention as a function of temperature and gradient steepness

On the basis of the above observations, the empirical relationship Eq. (11a) appeared to provide a somewhat better basis than Eq. (11) for predicting t_R as a function of temperature. This conclusion was confirmed by comparing errors in predicted retention times for the miscellaneous drugs of Table 3 in the 20 min gradient runs. Retention times for the runs at 30 and 66.3°C were used as input data. Using Eq. (11), these errors for other temperatures were as follows: ±0.18, 0.18 and 0.11 min (1 S.D.) for 39.5, 48.5 and 57.7°C, respectively. Using Eq. (11a), resulting errors were much smaller and essentially insignificant: ±0.04, 0.04 and 0.02 min for 39.5, 48.5 and 57.7°C, respectively. Similar comparisons for the other samples of Tables 2–5 also gave better agreement with Eq. (11a) vs. Eq. (11).

The procedure we propose (based on Eq. (11a)) for estimating retention time and optimizing separation as a function of gradient steepness and temperature is illustrated in Fig. 2. First, two runs are carried out at temperature T_a with gradient times t_{G1} and t_{G2} (top two circles of Fig. 2). A second two runs are carried out at temperature T_b with the same

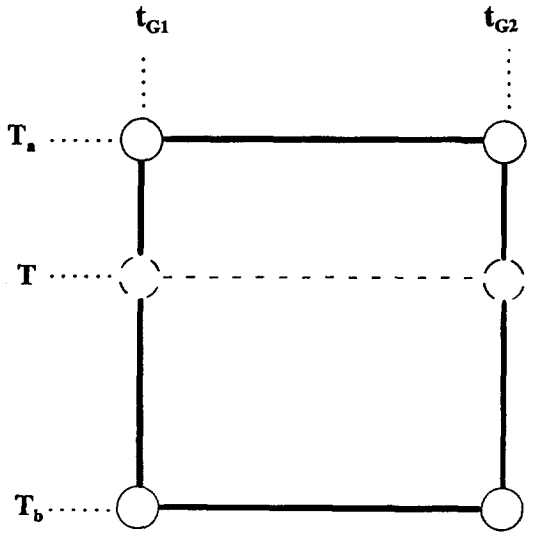


Fig. 2. Diagram illustrating the experimental design and the procedure for estimating retention times as a function of column temperature T and gradient time t_G. See Section 3 for details.

two gradient times (bottom two circles of Fig. 2). Now, for any other temperature T, Eq. (11a) is used to estimate values of t_R for gradient times t_{Ga} and t_{Gb} (represented by the two circles labeled T in Fig. 2). From the two values of t_R at any temperature T, values of S and k_w can be obtained for that temperature. This procedure allows the calculation of t_R for any temperature and any gradient time, as well as for different gradient conditions (initial and final %B, gradient shape) and different column conditions.

The data from all five laboratories (Appendices I–V) were used with Eq. (11a) to predict gradient retention t_R as a function of temperature. Results from four laboratories (A–D) are summarized in Table 8; data for laboratory E are discussed separately. Retention time differences Δt_R for adjacent bands are also summarized in Table 8. Values of Δt_R are of most interest in method development, since resolution R_s is proportional to Δt_R, and errors in predicted values of Δt_R lead to corresponding errors in R_s. It is convenient to express errors in predicted values of Δt_R as equivalent errors in α^*, the separation factor for two adjacent bands in gradient elution. The error in α^* ($\delta[\alpha^*]$) can be related to the error in Δt_R ($\delta[\Delta t_R]$) (Appendix A) as

$$\delta(\log \alpha^*) = (\Delta\phi S/t_G)\,\delta(\Delta t_R) \qquad (12)$$

Table 8 summarizes average errors in α^* (Eq. (12)) for each study. These errors in α^* did not exceed 2% (Table 8) and are therefore acceptable, since this corresponds to errors in predicted values of R_s that are typically <0.3 units [30]. Representative examples of the prediction of retention via Eq. (11a) are shown in Table 9. The prediction of retention for a simultaneous change in gradient steepness and temperature is illustrated in Table 10 for both the herbicide and pharmaceutical samples. The average error in α^* is only 0.6% for the herbicides and 0.4% for the pharmaceuticals.

4.2. Some anomalous retention time–temperature relationships

In a few cases, an atypical dependence of t_R on temperature was found for the studies summarized in Table 8. These are illustrated in Fig. 3. Data-set 1 in Fig. 3 is for tripelennamine as solute, which exhibits an increase in retention as temperature increases. Similar behavior was noted for aniline solute 11 (Table 2) at pH 3.6, but not at other pH values. A retention–temperature relationship of this kind, although unusual, has no effect on the accurate prediction of retention as a function of temperature, as long as t_R varies linearly with T (Eq. (11a)).

Data-sets 2 and 3 of Fig. 3 for sulfamethazine as solute show a maximum in t_R at an intermediate temperature (but relatively small deviations from Eq. (11a)). Similar behavior was noted for anilines 10 and 12 of Table 3 at pH 3.6, and aniline 11 at pH 4.6. The pK_a values of anilines 10 and 12 are estimated to be about 4.0 in this system (see Table 2 of Part III [11]), while the pK_a value of aniline 11 is 4.3. This suggests that this anomalous retention behavior may be associated with partly ionized basic solutes. A similar behavior was not observed for partly ionized benzoic acids.

The incidence of $t_R - 1/T$ plots having a maximum as in Fig. 3 was infrequent in the present study, and the retention errors observed for these examples resulted in errors in α of ± 2–3%, vs. ± 1% for other compounds in this sample. Errors in $\alpha > 2$% border on being unacceptable for HPLC method development. However, in Part III [11] it will be argued that method development is best carried out either at low

Table 8
Summary of predictions of retention based on Eq. (11a)

Sample, conditions[a]	Average errors[b]								
	T_1			T_2			T_3		
	t_R	Δt_R	α^* (%)	t_R	Δt_R	α^* (%)	t_R	Δt_R	α^* (%)
Benzoic acids (Table 2); 32.1 and 69.7°C data used for input; $T_1 = 24.6$°C, $T_2 = 50.9$°C ($n = 8$)									
15-min gradients									
pH 2.6	0.04	0.02	0.6	0.07	0.01	0.3			
pH 3.2	0.12	0.04	1.3	0.08	0.02	0.6			
pH 3.7	0.06	0.03	1.0	0.07	0.03	1.0			
pH 4.3	0.02	0.03	0.9	0.04	0.05	1.6			
45-min gradients									
pH 2.6	0.13	0.13	1.4	0.13	0.03	0.3			
pH 3.2	0.16	0.12	1.3	0.13	0.11	1.2			
pH 3.7	0.08	0.12	1.3	0.09	0.13	1.4			
pH 4.3	0.09	0.15	1.6	0.09	0.13	1.4			
Average error in α^*			1.2%			1.0%			
Anilines (Table 1); 32.1 and 69.7°C data used for input; $T_1 = 24.6$°C, $T_2 = 50.9$°C ($n = 9$)									
25-min gradients									
pH 2.6	0.06	0.03	0.9	0.07	0.03	0.8			
pH 3.6	0.03	0.04	0.9	0.05	0.05	1.1			
pH 4.6	0.11	0.05	1.2	0.09	0.05	1.2			
pH 5.6	0.05	0.02	0.5	0.03	0.03	0.8			
75-min gradients									
pH 2.6	0.10	0.09	0.9	0.15	0.15	1.5			
pH 3.6	0.14	0.09	0.7	0.22	0.12	0.9			
pH 4.6	0.15	0.19	1.5	0.29	0.25	2.0			
pH 5.6	0.07	0.04	0.3	0.13	0.05	0.4			
Average error in α^*			0.9%			1.1%			
(Table 3); 30 and 66.3°C data used for input; $T_1 = 39.5$°C, $T_2 = 48.5$°C, $T_3 = 57.7$°C									
Nitroalkanes[c] *($n = 10$)*									
$t_G = 20$ min	0.04	0.01	0.3	0.05	0.02	0.6	0.03	0.02	0.6
$t_G = 60$ min	0.12	0.03	0.3	0.18	0.03	0.3	0.14	0.03	0.3
Average error in α^*			0.3%			0.5%			0.5%
Drugs[c] *($n = 48$)*									
$t_G = 20$ min	0.04	0.03	1.3	0.04	0.03	1.3	0.02	0.02	0.8
$t_G = 60$ min	0.08	0.04	0.6	0.04	0.06	0.8	0.10	0.06	0.8
Average error in α^*			1.0%			1.1%			0.8%
(Table 4); herbicides; 39.9 and 57.3°C data usedfor input; $T_1 = 48.4$°C ($n = 9$)									
10-min gradient				0.01	0.00	0.6%			
20-min gradient				0.01	0.01	0.6%			
30-min gradient				0.01	0.01	0.5%			
Average error in α^*						0.6%			
(Table 5); pharmaceutical intermediates; 35 and 75°C data used for input; $T_1 = 55$°C ($n = 9$)									
30-min gradient				0.04	0.03	0.6%			
60-min gradient				0.05	0.03	0.3%			
90-min gradient				0.10	0.05	0.3%			
Average error in α^*						0.4%			

[a] Temperatures for predicted values of t_R.
[b] Errors in predicted retention times (t_R), difference in retention times for adjacent bands (Δt_R), and equivalent errors in α^* (Eq. IV-2).
[c] Mobile phase not preheated.

Table 9
Examples of retention prediction as temperature is varied

Sample, conditions	Compound	Retention time t_R		Δt_R [a]	
		Experimental	Calculated	Experimental	Calculated
Benzoic acids, pH 2.6, 15-min gradient; input data for 32.1 and 69.7°C, calculate t_R for 50.9°C[b]					
	1	6.88	6.80	1.22	1.23
	2	8.11	8.02	1.80	1.78
	3	9.89	9.82	0.22	0.23
	4	10.12	10.04	1.30	1.28
	5	11.40	11.34	0.21	0.21
	6	11.61	11.55	0.30	0.30
	7	11.91	11.85	0.99	1.00
	8	12.91	12.84		
Average error in t_R			±0.07 min		±0.01 min
Average error in α^*				0.3%	
Anilines, pH 2.6, 60-min gradient; input data for 32.1 and 69.7°C, calculate t_R for 50.9°C[b]					
	9	3.06	3.13	1.75	1.79
	10	4.85	4.88	0.55	0.56
	11	5.41	5.43	3.46	3.66
	12	9.07	8.89	0.40	0.26
	13	9.33	9.29	4.64	5.08
	14	14.41	13.93	7.50	7.26
	15	21.67	21.43	9.33	9.25
	16	30.92	30.76	5.26	5.22
	17	36.14	36.02		
Average error in t_R			±0.15 min		±0.15 min
Average error in α^*				1.2%	

[a] Difference in t_R for adjacent bands (proportional to α^*).
[b] Compound in Table 2.

pH, where basic solutes are completely ionized, or at high pH where they are non-ionized.

More accurate predictions of retention can in principle be achieved by the use of initial experiments at three (or more) different temperatures, with initial values of t_R used to define a suitable fitting function; e.g. a quadratic function of temperature. Apart from the requirement for two additional experiments, our experience with similar functions for other variables (e.g. %B in isocratic elution) suggests that retention predictions would be improved for interpolated temperatures and made worse for extrapolated predictions.

4.2.1. The use of temperatures <25°C

Changes in α with temperature have been reported for the reversed-phase separation of certain polycyclic aromatic hydrocarbons. It has been found [31,32] that values of α are highly dependent on column type and temperature, and also vary to a lesser extent with solvent strength (%B or b). Selectivity further depends on the three-dimensional shapes of these molecules. One theory for these shape-selective separations is that selectivity is affected by restricted access of bulky solute molecules between the alkyl ligands of the stationary phase. This theory, if correct, represents an additional factor in the dependence of retention on temperature. A corollary is that Eq. (11a) might prove less reliable for systems of this type.

Shape-selective separations were investigated in the present study, with some of our results described in Part IV [12]. The dependence of retention on temperature for separations of this type is compared in this section with Eq. (11a). In order to better evaluate these shape-selectivity effects, previous workers have used a nine-component test-sample that contains compounds of quite different shape [33]. This sample is described in Table 6, where we have classified these compounds into groups A–D, based

Table 10
Examples of retention prediction as temperature and gradient steepness are varied simultaneously

(A) Herbicide sample (Table 4); retention data for 39.9 and 57.3°C temperature, gradient times of 10 and 30 min, used to predict retention at 48.4°C and 20-min gradient

Solute	t_R (min)			Δt_R (min)		
	Expt.	Pred.	Error[a]	Expt.	Pred.	Error
1	1.53	1.52	0.01	0.31	0.33	0.02
2	1.84	1.85	0.01	1.94	1.93	0.01
3	3.78	3.78	0.00	1.19	1.18	0.01
4	4.97	4.96	0.01	0.79	0.78	0.01
5	5.76	5.75	0.01	1.86	1.87	0.01
6	7.63	7.62	0.01	0.63	0.63	0.00
7	8.26	8.24	0.02	0.36	0.37	0.01
8	8.62	8.61	0.01	0.39	0.39	0.00
9	9.01	9.00	0.01			
Average error in t_R			±0.01			±0.01

(B) Pharmaceutical sample (Table 5); retention data for 35 and 75°C temperature, gradient times of 30 and 90 min, used to predict retention at 55°C and 60-min gradient

Solute	t_R (min)			Δt_R (min)		
	Expt.	Pred.	Error[a]	Expt.	Pred.	Error
1	8.99	9.16	0.17	2.03	2.00	0.03
2	11.02	11.16	0.14	1.71	1.73	0.02
3	12.73	12.89	0.16	1.79	1.72	0.07
4	14.52	14.61	0.09	4.26	4.32	0.06
5	18.78	18.93	0.15	4.56	4.60	0.04
6	23.34	23.53	0.19	6.59	6.57	0.02
7	29.93	30.10	0.17	1.73	1.70	0.03
8	31.66	31.80	0.14	16.09	16.07	0.02
9	47.75	47.87	0.12			
Average error in t_R			±0.15			±0.04

on their structural characteristics and dependence of retention on temperature. The retention for compounds in group C decreases relative to groups B and A, for a change from a monomeric column to a polymeric column. A similar trend is observed for a decrease in temperature for both monomeric and polymeric columns.

The sample of Table 6 was separated on two different columns at different temperatures (0–45°C) and gradient times. The two alkyl-silica columns, Zorbax C18 (monomeric) and Hypersil Green PAH (polymeric), are quite different in terms of shape selectivity. It was observed that some of these test compounds gave markedly nonlinear plots of t_R vs. T, as illustrated in the examples of Fig. 4. It was further found that errors in predictions of t_R vs. T for these solutes varied widely (Table 11), depending on the solute group (Table 6) and column type. Solutes from group A give unacceptable errors on both columns, while solutes from group B are a problem on the Hypersil column.

The failure of Eq. (11a) for some of the cases of Table 11 is believed to be atypical of most practical HPLC separations, since molecules of somewhat peculiar shape are involved. The data do suggest that the separation of extended solutes (i.e. solutes with large length to breadth [L/B] ratios) may be more problematic than other solutes, particularly with polymeric stationary phases. One class of compounds that contain such extended solutes is polycyclic aromatic hydrocarbons (PAH), where polymeric columns are usually preferred for PAH analy-

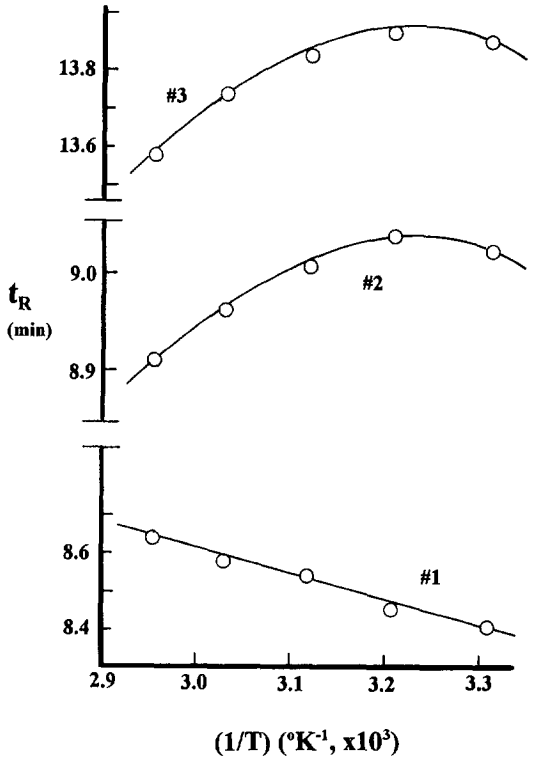


Fig. 3. Plots of retention time t_R vs. reciprocal temperature for some compounds from Table 3. 1 – Tripelennamine solute, 60-min gradient; 2 – sulfamethazine solute, 20-min gradient; 3 – sulfamethazine solute, 60-min gradient. Other conditions as for laboratory B (Section 3).

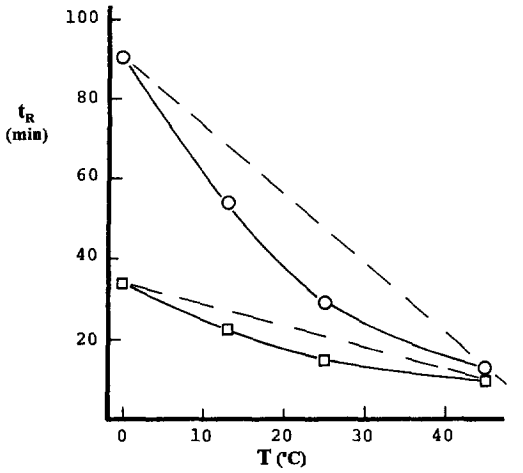


Fig. 4. Plots of retention time t_R vs. temperature for two compounds from the shape-selective text mixture. Conditions: 25×0.46 cm Hypersil Green PAH column; 1.0 ml/min; 80–100% ACN/water in 90 min; (○) 1,6-diphenylhexatriene; (□) pyrene.

ses. The temperature dependence of PAH separations is further examined in Part IV [12].

In the latter connection, other work [34] has demonstrated a frequent failure of Eq. (5) for temperature ranges that bracket 25°C. This has been attributed to a change in the conformation of C_8 or C_{18} bonded phases at a transition temperature of about 25°C although the existence of such phase transitions remains controversial. Eq. (5) was applicable either above or below this transition temperature. Other studies of C_{18}, C_{30} and C_{34} bonded phases show a higher transition temperature, and a failure of Eq. (5) at higher temperatures [35]; see also the discussion of [36].

Further work is needed to determine when Eq. (11a) may prove too imprecise for predicting retention for other sample types and/or conditions. On the basis of the present studies, however, we believe that Eq. (11a) will prove reliable for most practical HPLC separations at temperatures above 25°C. When the temperature range of interest includes both low (<25°C) and high temperatures (>35°), the accurate prediction of retention vs. temperature will probably require multi-point curve-fitting, as opposed to the two data-points used by Eq. (11a). A multi-point fit may also be preferred for basic solutes when pH≈ pK_a.

Useful temperatures for the experimental design of Fig. 2 should ideally bracket the temperature range of interest; $T_a = 30–40$°C and $T_b = 60–90$°C. If the mobile phase or sample/valve are not thermostated at the column temperature, then T_b should be <60°C.

4.3. Non-ideal temperature relationships

A deviation of measured from set-point temperatures was noted for some HPLC systems in Section 3. Because an approximately linear relationship exists between these two temperature scales, no error would have resulted from the use of set-point (instead of actual) temperatures to predict retention in the present study (based on Eq. (11a)). It seems likely that similar errors in set-point temperature for HPLC systems other than those studied here will also have little adverse effect on the accuracy of retention

Table 11
Error in predictions of retention vs. temperature for shape-selective sample of Table 6

Solute group	Solute	Error in predicted t_R (min)			
		Hypersil column			Zorbax column
		13°C		25°C	28°C
A	*p*-Terphenyl	9.0		11.0	–[a]
	1,6-Diphenyl-hexatriene	12.8		18.5	3.8
	Average error		12.8		3.8
B	Pyrene	4.3		5.4	0.7
	Triphenylene	7.5		8.8	1.3
	Average error		6.5		1.0
C	Tetraphenylmethane	−1.0		−0.2	−1.8
	1,3,5-Triphenyl-benzene	−0.1		1.2	−1.4
	Average error		−0.1		−1.6
D	Biphenyl	0.0		0.6	−0.4
	Triptycene	–[a]		–[a]	−0.9
	o-Terphenyl	−0.1		0.8	−1.1
	Average error		0.2		−0.8

Data for 0 and 45°C used as input. Other conditions: columns 25×0.46 cm; 1.0 ml/min; gradients of 60–100% ACN/water (Zorbax C18 column) and 80–100% ACN/water (Hypersil Green PAH column) in 90 min.
[a] Not measured.

time predictions. However, it should be noted that the transfer of a final, temperature-optimized method from one laboratory to another can lead to reproducibility problems if the set-point temperatures in the new or originating laboratories are different from measured temperatures, or if the extent of solvent preheating is not the same in the two laboratories.

An additional complication is possible if the mobile phase entering the column is not pre-heated so as to be at the desired temperature when it enters the column. The data of laboratory B were collected without pre-heating the mobile phase (by disconnecting the pre-heat line); we subsequently repeated these measurements for the 10 nitroalkane solutes with and without mobile phase preheating. Retention times for these two experiments are summarized in Table 12. As expected, preheating the mobile phase leads to a decrease in retention times for higher column temperatures, because the average column temperature is then higher. However, the prediction of retention as a function of observed temperature (using Eq. (11a)) was about as accurate for both cases (±0.05 min for no preheating, ±0.07 min for preheating) and predicted resolution (values of α) were equally accurate for both cases (±0.6%).

While an absence of mobile-phase preheating does not affect the accuracy of Eq. (11a), it has been observed that solute bands at elevated temperatures become much wider when preheating is omitted [37]. We observed this for the nitroalkane sample, as summarized in Table 13 and a similar behavior was seen for the benzoic acid sample when pre-heating was omitted. From a practical standpoint, therefore, mobile phase preheating is strongly recommended when column temperatures >50°C are used. Because the flow of solvent through a wide-diameter (>2 mm) HPLC column generates heat, minimum band widths are favored by the use of slightly lower temperatures for the incoming solvent vs. that of the column [36,38,39].

5. Conclusions

The present study has shown that gradient retention can be predicted with reasonable accuracy as a function of temperature T, using two experimental runs at different temperatures (all other conditions the same). Previous work [16,18] and the theory of gradient elution [9,13] have similarly shown that two experimental runs having different values of t_G can be used to predict retention time as a function of (a)

Table 12
Retention time predictions for nitroalkane solutes of Table 3

Solute	Retention time, t_R			
	Experimental			Calculated
	30.0°C	66.3°C	48.5°C	48.5°C
No mobile-phase pre-heat				
C_1 (1)	2.29	2.15	2.23	2.22
C_2 (2)	5.24	4.48	4.93	4.85
C_3 (3)	9.92	8.55	9.26	9.22
C_4 (4)	13.90	12.24	13.10	13.05
C_5 (5)	16.36	14.85	15.64	15.59
C_6 (6)	18.05	16.69	17.42	17.36
C_7 (7)	19.40	18.13	18.81	18.75
C_8 (8)	20.57	19.34	20.00	19.94
C_9 (9)	21.60	20.39	21.04	20.98
C_{10} (10)	22.53	21.31	21.96	21.91
Average error in predicted t_R = 0.05 min				
Average error in predicted α = 0.6%				
Mobile-phase pre-heat				
C_1	2.30	2.09	2.22	2.19
C_2	5.26	4.16	4.79	4.70
C_3	9.95	8.06	9.06	8.99
C_4	13.93	11.75	12.85	12.82
C_5	16.38	14.33	15.4	15.34
C_6	18.08	16.22	17.22	17.13
C_7	19.43	17.70	18.62	18.55
C_8	20.60	18.93	19.83	19.75
C_9	21.63	20.01	20.89	20.80
C_{10}	22.56	20.95	21.81	21.74
Average error in predicted t_R = 0.07 min				
Average error in predicted α = 0.6%				

Data for T = 30.0 and 66.3°C used for input; retention times for T = 48.5°C predicted.

Table 13
Bandwidth as a function of temperature, with and without mobile phase preheating

Solute	Bandwidth at half-height (min)		
	30.0°C	48.5°C	66.3°C
No mobile phase pre-heat			
C_1	0.059	0.065	0.080
C_2	0.085	0.097	0.153
C_3	0.098	0.123	0.155
C_4	0.079	0.115	–
C_5	0.069	0.105	0.146
C_6	0.066	0.099	0.139
C_7	0.065	0.097	0.138
C_8	0.067	0.095	0.139
C_9	0.063	0.091	0.131
C_{10}	0.062	0.088	0.128
Mobile phase pre-heat			
C_1	0.060	0.055	0.051
C_2	0.086	0.090	0.072
C_3	0.097	0.084	0.077
C_4	0.077	0.074	–
C_5	0.067	0.066	0.067
C_6	0.065	0.062	0.062
C_7	0.063	0.061	0.061
C_8	0.066	0.065	0.062
C_9	0.061	0.060	0.057
C_{10}	0.061	0.059	0.058

Nitroalkane solutes of Table 3, other conditions as in Table 12.

gradient time t_G, (b) other gradient conditions, and (c) column conditions (column dimensions, flow-rate and particle size). This means that predictions of retention can be made as a function of T, gradient conditions (including t_G), and column conditions by means of the experimental design of Fig. 2 (e.g. as in Table 10). This in turn allows the optimization of separation as a function of T and t_G. One exception should be noted, however. The use of polymeric-silane columns with certain samples can result in unacceptable errors in predicted retention times (Table 11) for temperatures <25°C; similar errors can occur for longer-chain bonded phases (>C_{18}) at temperatures >25°. Similarly, predictions of retention as a function of temperature appear to be less accurate (but marginally acceptable for method development) in the case of basic solutes that are partially ionized (where pH≈pK_a).

Following papers will demonstrate that the procedure of Fig. 2 can provide an efficient and simple approach to HPLC method development for most samples. While the main emphasis in this paper has been on the use of gradient conditions, the four experimental runs of Fig. 2 should also allow reliable predictions of isocratic separation as a function of temperature and solvent strength (%B) [3].

For method transfer, the separation conditions need to be meticulously documented for gradient methods run at elevated temperatures. Unless the temperature settings are calibrated, the nominal temperature setting of the column oven or thermostat can be in error by several degrees, and this can significantly affect the separation. Similarly, it is important to preheat the solvent to the column temperature before it enters the column. Finally, the system dwell volume must be specified.

6. Glossary of terms

Symbols used in this and the following three papers [10–12] are defined here. Units are given where appropriate and reference is made to a defining equation when available (Eq. II-3 refers to Eq. 3 of Part II; Eq. I-4 refers to Eq. (4) of Part I, etc.).

a', b', A, B	constants for a given solute and some set of conditions (only temperature changes) (Eqs. I-5, 10)
ACN	acetonitrile
b	gradient steepness parameter (Eq. I-4)
B	organic solvent in an HPLC mobile phase (%B); also, temperature coefficient of Eq. (5)
F	flow-rate (ml/min)
G	gradient compression factor (Eq. I-9)
J	anomalous increase in W (gradient elution, Eq. I-9)
k	retention factor; k in isocratic elution equals $(t_R - t_o)/t_o$
k^*	effective value of k in gradient elution (Eqs. II-4, -5); value of k when the solute reaches the column midpoint
k_o	value of k in gradient elution at start of separation (for $\phi = \phi_o$)
k_{oa}, k_{ob}	values of k_o for temperatures T_a and T_b
k_w	value of k for water as mobile phase ($\phi = 0$) (Eq. I-1)
k_{w1}, k_{w2}	values of k_w for solutes 1 and 2
k_1, k_2	values of k for two adjacent solute bands 1 and 2 in a chromatogram
MeOH	methanol
MTBE	methyl-*tert.*-butylether
n	number of sample components
N	column plate number
PAH	polycyclic aromatic hydrocarbon
S	solute parameter equal (in isocratic elution) to $d(k)/d\phi$ (min) (Eq. I-1)
S_1, S_2	value of S for solutes 1 and 2
T	measured column temperature (°K unless stated otherwise)
T'	nominal (set-point) column temperature according to the temperature controller setting (°C)
T_a, T_b	different values of T; e.g. 30 and 70°C
t_D	gradient dwell time (min); the time it takes for the gradient to arrive at the column inlet
t_G	gradient time (min); the time during which ϕ is changing
t_o	column dead-time (min)
t_R	solute retention time (min)
$(t_R)_a$, $(t_R)_b$	values of t_R for temperatures T_a and T_b
$(t_R)_n$	normalized retention time for a given solute based on $(t_R)_n = 1$ for lowest temperature and 0 for highest temperature (Table 7 and related text)
V_m	column dead-volume (ml); equal to $t_o F$
W	baseline bandwidth (min)
α	separation factor, equal to k_2/k_1 (Eq. II-2)
α^*	value of α for gradient elution
α_a, α_b	values of α for different conditions a and b; a and b can refer either to %B or to temperature
δB	that part of a solute B-value that is irregular (Eq. II-14)
$\delta(\alpha^*)$	error in predicted value of α^* (Eq. I-12) for gradient elution; α replaces α^* for isocratic elution
δS	that part of a solute S-value that is "irregular"; see discussion of Figs. II-1 and -2
$\delta(\Delta t_R)$	that part of a change in t_R with temperature that is "irregular"; see discussion of Part II; also, an error in predicted values of Δt_R (Eq. A-1 of Part I [Appendix A])
ΔB	difference in B-values for two solutes 1 and 2
$\Delta \log \alpha^*$	a change in $\log \alpha$ due to a change in either b or T from value a' to b' (Eqs. II-3, -7, -10, -11, gradient elution)
$\Delta \log \alpha^*(b)$	value of $\Delta \log \alpha^*$ for a change in gradient steepness b
$\Delta \log \alpha^*(T)$	value of $\Delta \log \alpha^*$ for a change in temperature T
ΔS	difference in S-values for two adjacent bands, equal to $S_2 - S_1$
$\Delta(1/T)$	$(1/T_b) - (1/T_a)$
Δt_R	change in t_R due to a change in T;

also, difference between experimental and calculated values of t_R

$\Delta\phi$ change in ϕ during the gradient ($\phi_f - \phi_o$); also, difference in ϕ for two mobile phases a and b ($\phi_b - \phi_a$) (Eq. II-3)

ϕ volume fraction of B-solvent in mobile phase; equal to 0.01 %B

ϕ^* effective value of ϕ in gradient elution; value of ϕ at the column midpoint when the solute reaches the midpoint

ϕ_a, ϕ_b values of ϕ for two different mobile phases (a and b)

ϕ_f value of ϕ at the end of the gradient

ϕ_o value of ϕ at the start of the gradient

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Appendix A

Relationship of errors in predicted retention times to errors in α

Eq. (7a) gives a relationship between a change in t_R and values of k_o. A similar relationship can be derived between errors in predicted values of Δt_R ($\delta[\Delta t_R]$) and errors in predicted values of α ($\delta[\alpha]$):

$$\delta(\Delta t_R) = (t_o/b)\,\delta(\log \alpha) \qquad \text{(A-1)}$$

This ignores differences in S for the two adjacent bands, but for small values of α this leads to negligible error. Errors in predicted values of α as a result of errors in predicted retention times are then given as

$$\delta(\log \alpha^*) = (b/t_o)\,\delta(\Delta t_R) = (\Delta\phi\, S/t_G)\,\delta(\Delta t_R) \qquad \text{(A-2)}$$

The effect of errors in α on resolution in gradient elution can be obtained from the equation

$$R_s = (1/4)(\alpha^* - 1)\,N^{1/2}\,(k^*/[1 + k^*]) \qquad \text{(A-3)}$$

corresponding to the similar relationship for isocratic separation (where α replaces α^*, and k replaces k^*). As discussed in Ref. [30], errors in α^* as large as 3% lead to (tolerable) errors in R_s of no more than 0.3 units for typical HPLC conditions.

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