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Journal of Chromatography A, 892 (2000) 107–121

JOURNAL OF CHROMATOGRAPHY A

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Reversed-phase separation of achiral isomers by varying temperature and either gradient time or solvent strength

L.R. Snyder*, J.W. Dolan

LC Resources Inc., 26 Silverwood Court, Orinda, CA 94563, USA

Abstract

The separation of several isomer pairs of widely varied structure was studied as a function of changes in temperature, gradient time, mobile phase pH, column type (''monomeric'' vs. ''polymeric'' C_{18}-silica) and organic solvent (methanol vs. acetonitrile). General conclusions are drawn which may prove useful in future attempts at the separation of these and other achiral isomers.

Keywords: Temperature effects; Solvent strength; Mobile phase composition; Gradient elution; Computer simulation; Resolution; Stereoisomers; Positional isomers; Steroids; Polynuclear aromatic hydrocarbons; Fatty acid methyl esters; Benzoic acids; Anilines

1. Introduction

Previous studies [1–5] have shown that reversed-phase liquid chromatography (RPLC) with optimized conditions of temperature T and either gradient time t_G or solvent strength (percentage of organic solvent, B, in the mobile phase) is a convenient and effective means of separating most samples. If four experimental separations are carried out for different values of T and t_G (e.g., 35 and 65°C, 20 and 60 min), computer simulation allows the accurate prediction of separation as a function of T, t_G (gradient) or % B (isocratic) [4–6]. Resolution maps provided by computer simulation can then lead to the easy optimization of T and t_G or % B, as well as other conditions (initial and final % B in a gradient, column dimensions, particle size and flow-rate).

When an adequate separation of the sample does not result from the above approach, the problem can usually be traced to one of two possible sample characteristics: (a) ''complex'' samples containing 20 or more components which result in crowded chromatograms [7], or (b) the presence in the sample of compounds of quite similar molecular structure. Two compounds of similar structure are likely to co-elute for some value of T and t_G, and compounds with similar polar substitution tend to show similar changes in retention as T or t_G is varied [8]. Thus, the separation of mixtures containing such compounds is likely to be more difficult.

The problem of ''chromatogram crowding'' for samples with a large number of components has been examined [7]. One solution to this problem is to carry out two separations of the sample with different conditions, so that every analyte is resolved adequately in one run or the other. A total analysis of the sample can then be achieved by compositing results for the two runs. The latter approach has been described using two runs that differ in both temperature and gradient time [9].

Isomeric compounds represent an extreme exam-

*Corresponding author. Tel.: +1-510-2546-334; fax: +1-510-5242-386.

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00050-9

ple of structural similarity, and their separation is regarded as generally more difficult than for non-isomeric compounds. In this connection, RPLC has been observed to be less effective than normal-phase LC for the separation of achiral isomers [10,11]. The RPLC separation of isomeric polycyclic aromatic hydrocarbons (PAHs) [12] and *cis–trans* carotenoids [13,14] has been shown to be favored by certain stationary phases (C_{18} columns made from polyfunctional silanes or C_{30} columns). The effect of a change in stationary phase on the RPLC separation of isomeric PAHs can be mimiced to a certain extent by changes in % B and especially T [12]. The RPLC separation of *cis* from *trans* chalcones has been reported as a function of C_{18} columns from different manufacturers [15]. Other studies reviewed in Refs. [10,11] have shown that isomer separations by RPLC are generally improved by using cyclodextrin-bonded phases instead of alkyl-silica columns. Finally, the separation of enantiomeric isomers is generally favored at lower temperatures (Ch. 12 of Ref. [10]). We are unaware of any systematic studies which have been reported for changes in isomer separation as a result of simultaneous change in temperature and either gradient time or isocratic solvent strength (% B). Where ''isomers'' are referred to in the following discussion, we mean positional or stereo isomers having the same number and kinds of functional groups in the molecule; i.e., ethanol and dimethylether are not regarded as ''isomeric'' by this convention.

The effects of T and t_G on the gradient RPLC separation of isomers of widely different molecular structure are reported here. Associated contributions from the column, mobile phase pH and different organic solvents (''B-solvents'') to isomer resolution (and change in resolution as a function of T and t_G) are also examined, but in less detail.

2. Theory

Literature data were first collected for the separation of various isomers as a function of temperature T and gradient time t_G. Computer simulation [4–6] then permitted the prediction of resolution as a function of experimental conditions, including T and t_G. Resolution R_s depends on the average retention factor k, column plate number N, and separation factor α (p. 27 of Ref. [10]), but values of α are of primary interest here. Therefore, computer simulations were carried out with N equal to 10 000, and gradient times were selected to give values of k^* (retention factor in gradient elution; see Ref. [16] or Ch. 8 of Ref. [10]) equal to either 2 or 8. Similarly, a difference in temperature of 25°C was used to measure the effect of T on resolution. Assuming an average value of $k^* \approx 4$, values of α can be related to resolution R_s reported here as:

$$\alpha \approx 1 + 0.05R_s \quad (1)$$

The separation of two compounds will be essentially the same, using either isocratic or gradient elution, when experimental conditions are the same (''corresponding conditions'') except for the variation of % B during gradient elution. This assumes that gradient conditions are selected so that isocratic and gradient retention (k and k^*) are the same [16]:

$$k^* = 0.85t_G F/(V_m \Delta\varphi S) \quad (2)$$

Here, F is flow-rate, V_m is the column dead volume, $\Delta\varphi$ is the change in the volume fraction φ of the B-solvent during the gradient, and S is a function of the two solutes and other experimental conditions. Therefore, values of R_s reported here for gradient elution (and related values of α from Eq. (1)) will be similar for corresponding isocratic separations. This is also true for corresponding *changes* in R_s with T or t_G. See Ref. [16] for a further discussion.

3. Experimental

The results cited here are based on previously published experimental data [2,4,5] that are referenced in later tables. DryLab for Windows software (LC Resources, version 2.05) was used for predictions of resolution as a function of T and t_G, assuming that $N = 10\,000$. Elsewhere [7] it has been shown that such predictions are expected to be accurate to within ± 0.2 R_s units. Similarly, all chromatograms shown here are computer simulations based on the experimental data of Refs. [2,4,5]. While no new data are reported in this paper, this is the first time that isomer separations (as a function of

T and t_G) from several prior studies have been examined for the purpose of drawing general conclusions.

4. Results and discussion

4.1. Neutral isomers

Table 1 summarizes values of R_s for several neutral isomers as a function of T and t_G. Exact values of T and t_G are given in Table 1 (footnotes), but these are of less concern. Of major interest are *maximum* possible values of R_s for optimized T and t_G, and *changes* in R_s (ΔR_s) as a result of changing gradient time by a factor of 4 and temperature by 25°C. The latter results are listed in Table 1. Note that observed values of R_s for intermediate values of T or t_G were never significantly larger than the maximum values of R_s shown in Table 1.

4.1.1. Hydroxytestosterones

Consider first the separation of the 14 hydroxytestosterone isomers of Table 1. Each of these compounds is substituted by two hydroxyl and one carbonyl groups. Thus, we are dealing with a group

Table 1
Separation of neutral isomers on monomeric C_{18} columns; t_{G1} chosen to give $k^*\approx 2$, t_{G2} to give $k^*\approx 8$[a]

Isomer pairs[b]	Isomer type[c]	R_s				ΔR_s[d]		Maximum ΔR_s
		T_1, t_{G1}	T_2, t_{G1}	T_1, t_{G2}	T_2, t_{G2}	δT	δt_G	
Corticosteroids [4][e]								
20-Dihydroprednisolone/cortisone	Positional	8.4	7.3	**8.5**	7.4	−1.1	0.1	1.2
Betamethasone/dexamethasone	Stereo	1.2	1.2	**1.7**	1.6	0.1	0.4	0.5
Hydroxytestosterones [4][f]								
7α/6α	Positional	1.8	0.9	**2.6**	0.9	−1.3	0.4	1.7
6α/15α[i]	Positional	−0.2	**1.9**	1.1	0.2	0.6	−0.2	2.1
15α/15β[i]	Positional	2.6	0.6	3.5	**3.6**	−1.0	1.9	2.9
15β/19	Positional	0.3	**0.5**	−0.2	0.4	0.4	−0.3	0.7
19/6β	Positional	0.1	0.1	−0.7	−0.3	0.2	−0.6	0.8
6β/14α	Positional	0.5	1.2	0.6	**1.6**	0.8	0.2	1.1
14α/11α	Positional	1.4	1.1	**3.7**	3.0	−0.5	2.1	2.6
11α/16α	Positional	1.4	0.6	**2.2**	1.1	−1.0	0.7	1.6
16α/2β	Positional	5.6	6.1	6.0	**6.9**	0.7	0.6	1.3
2β/2α	Stereo	**0.4**	0.0	0.2	0.1	−0.2	−0.1	0.4
2α/11β	Positional	0.8	0.4	**0.9**	0.0	−0.6	−0.2	0.9
11β/18	Positional	3.7	3.6	**4.7**	3.6	−0.6	0.5	1.1
18/16β	Positional	0.6	0.6	**0.8**	0.8	0.0	0.2	0.2
Other								
Phenanthrene/anthracene [2][g]	Positional	2.7	1.6	**3.4**	2.9	−0.8	1.0	1.8
Fluoranthene/pyrene [2][g]	Positional	2.1	1.9	**2.4**	2.1	−0.3	0.3	0.5
LSD-1578/LSD-1583 [4][h]	Stereo	0.6	0.6	0.9	**1.1**	1.1	0.4	0.5

[a] For testosterone sample (more than one isomer pair), only adjacent isomer bands shown ($t_G = 17$ min, $T = 35$°C). Maximum R_s for each isomer pair shown in bold. Values of ΔR_s refer to the maximum change in R_s for the allowed change in temperature (25°C) or k^* (approximately a factor of [8/2] = 4). Maximum values of ΔR_s (last column) refer to the maximum change in R_s as a result of simultaneous change in temperature and k^*. Based on data of Refs. [4,5].

[b] Isomers listed in order of elution i, j for T_1, t_{G1}; $\alpha = k_j/k_i$.

[c] Stereo = stereochemical isomer.

[d] Change in R_s for a change in T (δT) by 25°C or t_G (δt_G) by a factor of 4.

[e] Conditions: 25×0.46 cm C_{18} column; 0–100% acetonitrile in water; 2.0 ml/min ($t_G = 20$, 80 min, $T = 30$, 55°C).

[f] Conditions: 25×0.46 cm C_{18} column; 0–80% acetonitrile in water; 2.0 ml/min ($t_G = 14$, 56 min, $T = 28$, 53°C).

[g] Conditions: 25×0.46 cm C_{18} column; 50–100% acetonitrile in water; 2.0 ml/min (20–80 min, 23–48°C).

[h] Conditions: 25×0.46 cm C_{18} column; 5–80% acetonitrile in water; 1.0 ml/min (21–84 min, 35–60°C).

[i] Approximate values because of peak overlap in orginal experimental separations.

of compounds which have significant polar substitution, a fact that will prove relevant to our later discussion. Fig. 1 shows the separation of these isomers as a function of T and t_G; a cursory examination shows that changes in T or t_G can result in significant changes in selectivity. For example, a 25°C increase in T (Fig. 1b vs. a) results in improved separation of 6α and 14α, but poorer separation of the 11α, 16α and 11β isomers. Likewise an increase in t_G by four-fold (Fig. 1c vs. a) provides better separation of 6α, 15α, 18 and 16β, but poorer separation of 14α. Fig. 2 shows a resolution map for the 14α/11α isomer pair of Table 1, with superimposed chromatograms for extremes in gradient time (14 and 56 min) and temperature (28 and 53°C). Maximum resolution occurs for lower temperatures and longer gradients.

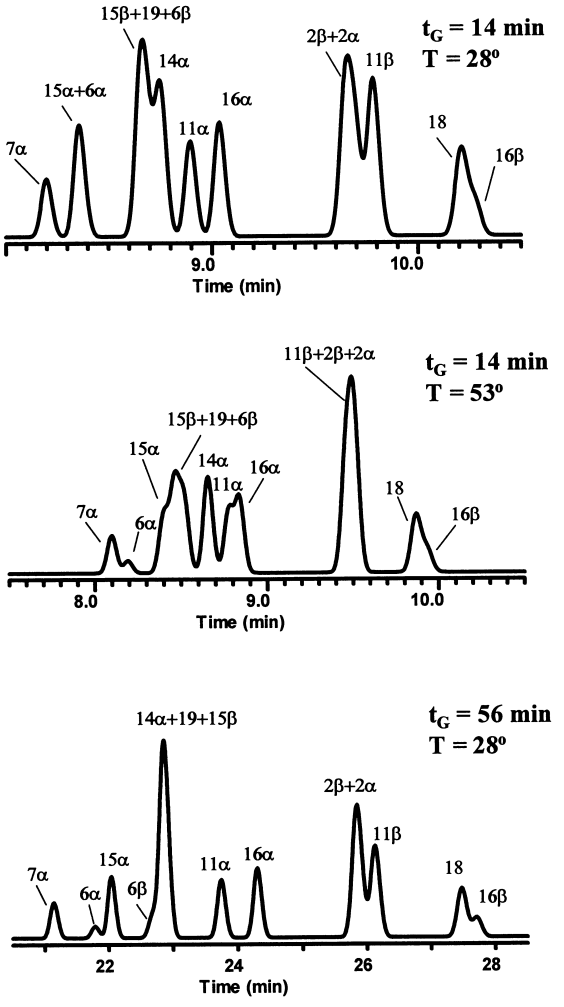

Fig. 1. Separation of 14 hydroxytestosterone isomers as a function of temperature T and gradient time t_G. Other conditions as in Table 1. Separations shown are computer simulations based on the experimental data of Ref. [4].

The resolution values (R_s) of Table 1 for the hydroxytestosterones are for 13 *adjacent* peak pairs which are defined by a separation with $t_G = 14$ min ($k \approx 2$) and $T = 28$°C (as in Fig. 1a). However, there are 78 other isomer pairs; the maximum resolution of each of these 91 isomer pairs (by varying T by 35°C and t_G by four-fold) is summarized in Table 2. We note first that only 6 out of 91 isomer pairs cannot be separated with $R_s \geq 1$, when T and t_G are optimized. This is somewhat surprising, in view of the common perception that RPLC is often ineffective as a means for the separation of isomers.

Another conclusion from Table 2 concerns preferred conditions of T and t_G for the separation of this group of isomers. Resolution generally improves for an increase in t_G, because of a corresponding increase in k^* (Ch. 8 of Ref. [10]). Likewise, other studies have shown a generally better resolution of isomers at lower temperatures. Conditions for maximum resolution of the isomer pairs of Table 2 are given in Table 3. Table 4 summarizes these preferred conditions, where we see that longer gradient times and lower temperatures are generally preferred, but *higher* temperatures and/or *shorter* gradients give greater resolution for one out of three hydroxytestosterone isomer pairs.

The relative change in resolution (ΔR_s) for an increase in T (δT) or t_G (δt_G) is listed in Table 1 for these neutral (adjacent) isomers. Increases in T or t_G may either increase or decrease resolution, as noted by the sign for values of ΔR_s in Table 1. The average *absolute* value of ΔR_s is 0.6 for a change in T by 25°C and 1.0 for a change in t_G by a factor of 4. Thus, as found previously [8], changes in T are less effective than are changes in t_G or % B as a means of changing values of α and thereby maximizing resolution. However, change in either T or t_G is likely to have a significant effect on resolution.

Maximum values of ΔR_s as a result of change in *both* T and t_G are also listed in Table 1 for these isomer pairs. The average of these values is ΔR_s (T, t_G) = 1.3±0.8. Thus, *simultaneous* change in T and

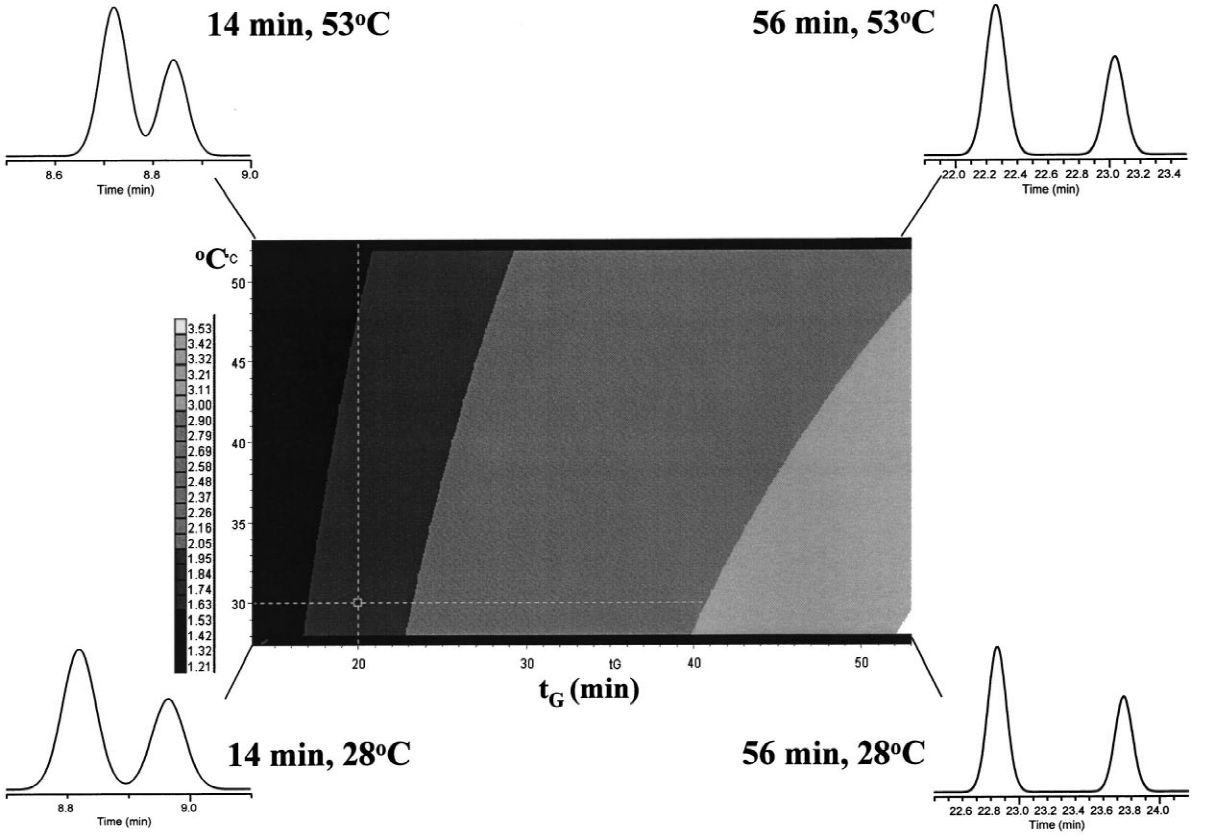

Fig. 2. Resolution map and predicted chromatograms for separation of 14α- and 11α-hydroxytestosterone isomers as a function of temperature and gradient time. Other conditions as in Table 1.

Table 2
Maximum resolution for separation of all hydroxytestosterone isomers from each other; $T_1 = 28°C$, $T_2 = 53°C$, t_{G1} to give $k^* \approx 2$, t_{G2} to give $k^* \approx 8$[a]

	7α	15α	6α	15β	19	6β	14α	11α	16α	2β	2α	11β	18	16β
7α	–	2.6	3.8	4.4	7.0	6.2	6.8	10.6	12.9	18.1	18.2	19.0	23.6	24.4
15α		–	1.9	4.6	4.3	4.0	5.5	8.7	10.2	16.2	16.3	16.4	21.1	21.9
6α			–	3.6	3.9	3.6	5.1	8.1	9.4	15.3	15.4	15.8	20.6	21.4
15β				–	**0.5**	**0.9**	1.8	4.9	5.9	12.4	12.5	12.6	17.0	17.8
19					–	**0.7**	1.3	4.4	5.8	11.9	12.0	12.3	17.0	17.8
6β						–	1.6	4.6	6.4	12.1	12.7	12.8	17.4	18.2
14α							–	3.6	5.8	12.1	11.3	12.1	16.8	17.6
11α								–	2.2	7.9	8.1	9.0	13.8	14.6
16α									–	6.9	6.9	6.9	11.7	12.6
2β										–	**0.4**	1.2	5.8	6.6
2α											–	**0.9**	5.5	6.3
11β												–	4.7	8.8
18													–	**0.8**
16β														–

[a] Bold values are for $R_s < 1$.

Table 3
Conditions for maximum resolution of band pairs shown in Table 2[a]

	7α	15α	6α	15β	19	6β	14α	11α	16α	2β	2α	11β	18	16β
7α	–	HL	HL	HL	HL	HL	HL	HL	HL	HL	HL	HL	HL	HL
15α		–	LH	HL	HL	HH	HH	HH	HL	HH	HH	HL	HL	HL
6α			–	HL	HH	HH	HH	HH	HL	HH	HH	HL	HL	HL
15β				–	LH	HL	LH	HH	HH	HH	HH	HH	HL	HL
19					–	HL	LH	HH	HL	HH	HH	HL	HL	HL
6β						–	HH	HH	HL	HH	HH	HL	HL	HL
14α							–	HL	HL	HL	HL	HL	HL	HL
11α								–	HL	HL	HL	HL	HL	HL
16α									–	HH	HH	HL	HL	HL
2β										–	LL	LL	HL	HL
2α											–	HL	HL	HL
11β												–	HL	HL
18													–	HH
16β														–

[a] First letter (H, high; L, low) is for t_G, second letter is for T; e.g., HL indicates a high value of t_G (i.e., t_{G2}) and a lower value for T (i.e., T_1).

t_G is able to create even larger changes in the resolution of these adjacent isomers. A correlation between maximum values of R_s and values of ΔR_s (T, t_G) is expected, since larger values of either of these two quantities implies larger differences in the interactions of the two isomers with the mobile and/or stationary phases. However, the observed correlation is relatively weak:

$$\Delta R_s\,(T, t_G) = 0.9 + 0.20R_s(\text{max});$$
$$r = 0.44, \text{ std. error} = 0.7$$

Table 4
Summary of preferred conditions for separating isomer pairs or samples composed of isomers[a]

Isomers	Conditions for maximum R_s			
	LL	LH	HL	HH
Hydroxytestosterones (Table 1)	2	4	59	26
Other neutral isomers (Table 1)	0	0	4	1
Fatty acid methyl esters (Table 5)	0	1	16	1
Substituted benzoic acids (Table 7)	0	2	15	0
Total	2	7	94	28
%	2	5	72	21

[a] Data of Tables 1, 2, 5 and 7. First letter (H, high; L, low) is for t_G, second letter is for T; e.g., HL indicates a high value of t_G and a lower value for T.

While small values of R_s(max) necessarily signify small values of ΔR_s (T, t_G), the reverse is not necessarily so.

The data of Table 1 suggest that stereoisomers are generally less well resolved than are positional isomers; average values of R_s(max) = 3.0 (positional isomers) vs. 1.1 (stereoisomers). However, the data of Table 1 only include adjacent isomers. There are three additional hydroxytestosterone stereoisomer pairs (Table 2: 6α/6β, 11α/11β, 16α/16β), all of which have values of R_s(max) > 2. A tentative conclusion for the isomers of Tables 1 and 2 is that positional isomers will be generally easier to separate than stereoisomers, but isomers of either type are likely to be resolvable by RPLC when T and t_G are optimized.

Although it is possible to separate most testosterone isomer pairs from each other, the simultaneous separation of more than two isomers will generally prove more difficult. This can be illustrated as follows. Returning to Table 2, the removal of four isomers (15β, 19, 2β, 18) from the testosterone sample leaves 10 compounds for which $R_s > 1$ when T and t_G are optimized for each isomer pair. The separation of this 10-component mixture is shown as a function of T and t_G in the resolution map of Fig. 3a. Maximum resolution ($R_s = 0.7$) results for $t_G = 42$ min and $T = 31$°C (Fig. 3b). Because optimized

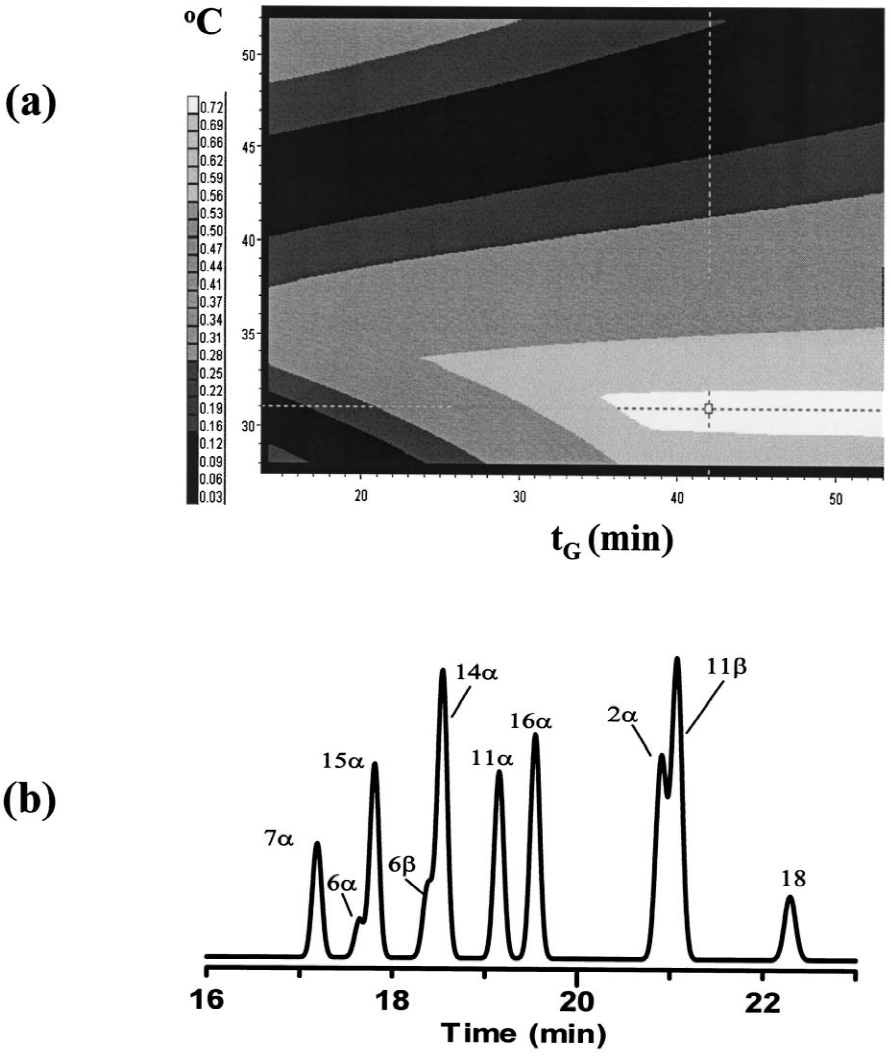

Fig. 3. Separation of 10 hydroxytestosterone isomers (compounds of Table 1, excluding 15β, 19, 2β, 18 isomers. (a) Resolution map as a function of T and t_G; (b) optimized separation for $T=31$°C and $t_G=42$ min. Separations shown are computer simulations based on the experimental data of Ref. [4].

conditions differ for each isomer pair, it is not possible to achieve $R_s>1$ for the entire sample in a single run with defined values of T and t_G. The latter generalization may contribute to the perception that RPLC is often ineffective in the separation of isomers. That is, samples which contain several isomeric compound pairs are likely to be challenging, but the separation of any single isomeric pair of compounds is likely to be fairly easy by optimizing T and t_G.

4.1.2. Octadecanoic acid methyl esters. Effects of solvent and column on isomer separation

Previous studies [12–14] have shown that the use of different C_{18} columns can result in large changes in the separation of certain isomeric compounds

(PAHs, carotenoids) that differ in molecular shape. So-called ''polymeric'' columns made from polyfunctional silanes bonded to silica provide enhanced shape selectivity, which has been attributed to the presence of ''slots'' in the stationary phase that restrict the access of bulkier isomers. Fig. 4 summarizes the separation of four isomers of methyloctadeceneoate as a function of column type, the mobile phase B-solvent, gradient time and temperature. The separations of Fig. 4 are for (a) a monomeric C_{18} column and acetonitrile as B-solvent, (b) a monomeric C_{18} column and methanol as B-solvent, and (c) a polymeric C_{18} column and acetonitrile as B-solvent. In each case, the best separation occurs for a longer gradient time and a lower temperature. Similarly, a polymeric column and acetonitrile as B-solvent (Fig. 4c) provide improved resolution for this four-component mixture.

Tables 5 and 6 provide further detail for the separations of Fig. 4. The average maximum resolution of adjacent isomers (Table 5) is similar and marginal for the monomeric column and either acetonitrile (avg. $R_s = 0.6$) or methanol (avg. $R_s = 0.5$). That is, interactions of these compounds with both the mobile and stationary phase appear to be quite similar. Likewise, average changes in R_s as a result of change in temperature (0.0) and gradient time (0.2) are quite small. For use of a *polymeric* column and acetonitrile as B-solvent, the average resolution of these four isomers is significantly greater (avg. $R_s = 1.6$). This can be reconciled in terms of the greater shape selectivity of a polymeric

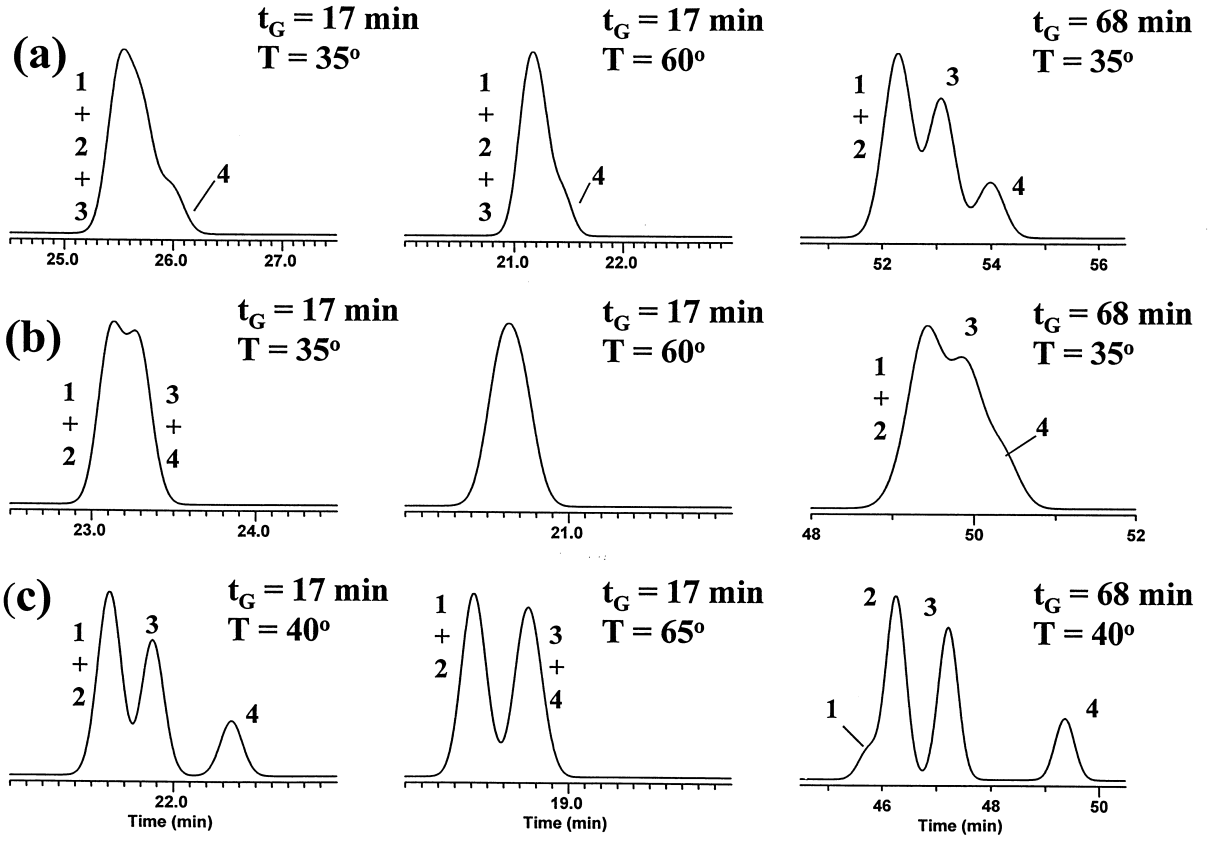

Fig. 4. Separation of four C_{18}-ene methyl fatty acid isomers as a function of temperature T and gradient time t_G. Sample: 1, *cis*-9-ene; 2, *cis*-7-ene; 3, *cis*-6-ene; 4, *trans*-9-ene. Other conditions as in Table 5. Separations shown are computer simulations based on the experimental data of Refs. [4,5].

Table 5
Separation of neutral fatty-acid-ester isomers on both monomeric and polymeric C_{18} columns, and with acetonitrile (ACN) or methanol (MeOH) as B-solvent[a]

Isomer pairs[b]	Isomer type[c]	R_s				ΔR_s (avg)[d]		Maximum ΔR_s
		T_1, t_{G1}	T_2, t_{G1}	T_1, t_{G2}	T_2, t_{G2}	δT	δt_G	
Methyloctadecanoate-mono-enes (ACN, monomeric)[e,f]								
cis-9-ene/*cis*-7-ene	Positional	0.2	**0.2**	0.2	0.2	0.0	0.0	0.0
cis-7-ene/*cis*-6-ene	Positional	0.5	0.4	**0.8**	0.7	−0.1	0.3	0.4
cis-6-ene/*trans*-9-ene	Positional and stereo	0.6	0.6	**0.9**	0.8	−0.0	0.3	0.3
Methyloctadecanoate-mono-enes (MeOH, monomeric)[e,g]								
cis-9-ene/*cis*-7-ene	Positional	0.0	0.2	0.3	**0.4**	0.1	0.2	0.4
cis-7-ene/*cis*-6-ene	Positional	0.5	0.3	**0.6**	0.5	−0.1	0.1	0.3
cis-6-ene/*trans*-9-ene	Positional and stereo	0.2	0.2	**0.6**	0.5	0.0	0.3	0.4
Methyloctadecanoate-mono-enes (ACN, polymeric)[e,h]								
cis-9-ene/*cis*-7-ene	Positional	0.1	0.2	**0.6**	0.4	−0.1	0.4	0.5
cis-7-ene/*cis*-6-ene	Positional	1.0	0.9	**1.3**	0.9	−0.3	0.2	0.4
cis-6-ene/*trans*-9-ene	Positional and stereo	1.6	0.1	**2.7**	1.1	−1.6	1.1	2.6

[a] Maximum R_s for each isomer pair shown in bold. Values of ΔR_s refer to the maximum change in R_s for the allowed change in temperature (25°C) or k^* (approximately a factor of [8/2]=4). Maximum values of ΔR_s (last column) refer to the maximum change in R_s as a result of simultaneous change in temperature and k^*. Based on data of Refs. [4,5].

[b] Isomers listed in order of elution i, j for T_1, t_{G1}; $\alpha = k_j/k_i$.

[c] Stereo = stereochemical isomer.

[d] B-solvent is either ACN or MeOH; monomeric and polymeric refer to bonded phase.

[e] Monomeric refers to columns prepared from monofunctional silanes; polymeric refers to columns prepared from polyfunctional silanes (pp. 189–190 of Ref. [10]).

[f] Conditions: 25×0.46 cm C_{18} column; 50–100% ACN in water; 1.0 ml/min (17–68 min, 35–60°C).

[g] Conditions: 25×0.46 cm C_{18} column; 75–100% MeOH in water; 1.0 ml/min (17–68 min, 35–60°C).

[h] Conditions: 25×0.46 cm C_{18} column; 60–100% ACN in water; 1.0 ml/min (17–68 min, 40–65°C).

Table 6
Maximum resolution for separation of adjacent C_{18}-ene methyl fatty acid isomers from each other; $T_1 \approx 35$°C, $T_2 \approx 60$°C, t_{G1} to give $k^* \approx 2$, t_{G2} to give $k^* \approx 8$[a]

Conditions	Solute	Maximum R_s			
		cis-9-ene	*cis*-7-ene	*cis*-6-ene	*trans*-9-ene
Monomeric column, acetonitrile as B-solvent	*cis*-9-ene	–	**0.2**	1.0	2.0
	cis-7-ene		–	**0.8**	1.7
	cis-6-ene			–	**0.9**
	trans-–9-ene				–
Monomeric column, methanol as B-solvent	*cis*-9-ene	–	**0.4**	**0.9**	1.5
	cis-7-ene		–	**0.6**	1.1
	cis-6-ene			–	**0.6**
	trans-9-ene				–
Polymeric column, acetonitrile as B-solvent	*cis*-9-ene	–	**0.6**	2.0	4.7
	cis-7-ene		–	1.3	4.1
	cis-6-ene			–	2.7
	trans-9-ene				–

[a] Bold values are for $R_s < 1$. See Table 5 for other details.

vs. monomeric column, coupled with the effect on molecular shape of the differences in structure for the compounds of Table 5. Likewise, average changes in R_s as a result of change in temperature (0.7) and gradient time (0.6) are much larger than for the monomeric column (0.0 and 0.2, respectively). We believe that these lower values of R_s and ΔR_s (compared to values for the hydroxytestosterones) may result from an absence of more polar substituents on the solute molecule.

Table 6 expands the data of Table 5 to include all fatty-acid-ester isomer pairs (not just adjacent bands). Only 10 out of 18 such pairs are resolved with $R_s > 1$, which may reflect the apparently small difference in interaction of these structurally very similar solutes with the mobile vs. stationary phases. Table 7 lists preferred conditions for the separation of each isomer pair, and these data are summarized in Table 5. Again, there is a strong preference for longer gradient times and lower temperatures, with a few exceptions.

4.2. Isomeric acids and bases

Table 8 summarizes the separation of several adjacent isomers of substituted benzoic acids and anilines as a function of mobile phase pH. Chromatograms for the separation of the chloroaniline isomers are shown in Fig. 5. Maximum resolution for any one of these isomers pairs at any pH is never less than $R_s = 1.4$, if T and t_G are optimized for that pair of compounds. Similarly, maximum changes in resolution ΔR_s for the 17 combinations of isomer pair and mobile phase pH in Table 8 are equal to 4.3 ± 4.0 units in R_s. That is, changes in T and t_G are capable of creating large changes in resolution for these particular examples, but the effects of T and t_G on resolution vary widely with the isomer pair and especially pH. Absolute changes in resolution with t_G (avg. $\Delta R_s = 3.4$) are much larger than for change in in R_s with T (avg. $\Delta R_s = 0.6$).

Further insight into the resolution changes summarized in Table 8 is possible by considering the relative ionization of the sample. The latter can be inferred from the mobile phase pH and sample pK_a values. Values of pK_a can be estimated from values for a comparable mobile phase system (25–35% methanol in buffer, vs. present acetonitrile in buffer systems) for which sample retention (k vs. k^*) is similar to that in Table 8; these estimates of pK_a for the system of Table 8 are given in Table 9. Similar values (± 0.1–0.2 units) of pK_a for these same compounds have been observed in acetonitrile in buffer gradients with $k^* \approx 10$ [18]. Fig. 6 shows plots of R_s for $t_G = 48$ min and $T = 35$°C vs. pH for three different isomer pairs: (a) 3,4- and 3,5-dich-

Table 7
Conditions for maximum resolution of band pairs shown in Table 6[a]

Conditions	Solute	Maximum R_s			
		cis-9-ene	*cis*-7-ene	*cis*-6-ene	*trans*-9-ene
Monomeric column, acetonitrile as B-solvent	*cis*-9-ene	–	LH	HL	HL
	cis-7-ene		–	HL	HL
	cis-6-ene			–	HL
	trans-9-ene				–
Monomeric column, methanol as B-solvent	*cis*-9-ene	–	LH	HL	HL
	cis-7-ene		–	HL	HL
	cis-6-ene			–	HL
	trans-9-ene				–
Polymeric column, acetonitrile as B-solvent	*cis*-9-ene	–	HL	HL	HL
	cis-7-ene		–	HL	HL
	cis-6-ene			–	HL
	trans-9-ene				–

[a] First letter (H, high; L, low) is for t_G, second letter is for T; e.g., HL indicates a high value of t_G and a lower value for T. See Table 5 for other details.

Table 8
Separation of acids and bases on monomeric C_{18} columns as a function of mobile phase pH (pH is held constant for each separation)[a]

Isomer pairs[b]	Isomer type[c]	R_s				ΔR_s[d]		Maximum ΔR_s
		T_1, t_{G1}	T_2, t_{G1}	T_1, t_{G2}	T_2, t_{G2}	δT	δt_G	
Benzoic acids[e]								
2-Nitro/3-nitro, pH 2.6	Positional	13.9	13.8	**23.2**	20.7	−1.3	8.1	9.3
2-Fluoro/3-fluoro, pH 2.6	Positional	7.7	7.7	**11.4**	10.9	−0.3	3.5	3.7
2-Fluoro/3-fluoro, pH 3.2	Positional	9.7	9.7	**14.2**	13.5	−0.3	4.2	4.5
2-Fluoro/3-fluoro, pH 3.7	Positional	12.5	13.1	**18.8**	17.5	−0.4	5.4	5.7
2-Fluoro/3-fluoro, pH 4.3	Positional	9.9	13.2	**25.8**	23.3	0.4	13.0	15.9
Anilines[f]								
4-Cl/3-Cl, pH 2.6	Positional	13.3	11.1	**13.1**	12.1	−1.6	0.4	2.0
4-Cl/3-Cl, pH 3.6	Positional	3.5	2.8	**7.2**	5.5	−1.2	3.2	4.4
4-Cl/3-Cl, pH 4.6	Positional	2.2	1.8	**3.0**	2.4	−0.5	0.7	1.2
4-Cl/3-Cl, pH 5.6	Positional	2.0	1.7	**2.5**	2.1	−0.4	0.4	0.8
3-Cl/2-Cl, pH 2.6	Positional	7.7	6.6	**16.1**	13.0	−2.1	7.4	9.5
3-Cl/2-Cl, pH 3.6	Positional	1.7	1.7	**2.2**	2.2	0.0	0.5	0.5
3-Cl/2-Cl, pH 4.6	Positional	1.3	**1.4**	0.2	0.8	0.3	−0.8	1.0
3-Cl/2-Cl, pH 5.6	Positional	1.2	**1.4**	0.2	0.7	0.3	−0.9	1.2
3,4-di-Cl/3,5-di-Cl, pH 2.6	Positional	6.0	5.8	**10.0**	9.4	−0.4	3.7	4.2
3,4-di-Cl/3,5-di-Cl, pH 3.6	Positional	5.3	2.1	**7.6**	7.3	−0.2	2.3	5.5
3,4-di-Cl/3,5-di-Cl, pH 4.6	Positional	5.3	5.2	**7.3**	7.0	−0.2	1.9	1.9
3,4-di-Cl/3,5-di-Cl, pH 5.6	Positional	5.6	5.1	**7.4**	7.1	−0.4	1.9	2.3

[a] Maximum R_s for each isomer pair shown in bold. Based on data of Ref. [2].
[b] Isomers listed in order of elution *i*, *j* for T_1, t_{G1}; $\alpha = k_j/k_i$.
[c] Stereo = stereochemical isomer.
[d] Change in R_s for a change in T (δT) by 25°C or t_G (δt_G) by a factor of 4.
[e] Conditions: 15×0.46 cm C_{18} column; 5–50% ACN in buffer; 1.0 ml/min (12–48 min, 35–60°C).
[f] Conditions: 15×0.46 cm C_{18} column; 5–65% ACN in bufer; 1.0 ml/min (12–48 min, 35–60°C).

loroanilines; (b) 2- and 3-chloroanilines; (c) 2- and 3-fluorobenzoic acids. The arrows at the top of each figure indicate a pH range in which sample ionization changes significantly with pH.

The two aniline isomers for which data are plotted in Fig. 6a have estimated pK_a values of 2.0 and 2.3. Therefore, for pH > 3.5, these compounds are >97% in the non-ionized form, and resolution is not expected to be affected by changes in pH. Changes in resolution with T or t_G are essentially constant when pH > 3.5, which is also expected. A similar pattern is seen in Fig. 6b for 2- and 3-chloroaniline (average p$K_a \approx 2.5$), and in Fig. 6c for 2- and 3-fluorobenzoic acid (average p$K_a \approx 3.5$). However, in each of these three examples of Fig. 6, resolution and values of ΔR_s change dramatically with pH when $(pK_a - 1.5) < pH < (pK_a + 1.5)$; i.e., when sample ionization changes significantly with pH.

It is well known that a change in pH which changes sample ionization can have a large effect on the resolution of acids or bases, because *relative* ionization can change with pH – with a large effect on relative retention. Similarly, a change in either % B (equivalent to a change in t_G) or T can alter the *effective* pK_a value of the solute, which is then equivalent to a change in pH. This latter fact not only explains the large changes in values of ΔR_s observed in Fig. 6, but it also accounts for the larger absolute values of ΔR_s in Table 8 (vs. corresponding values in Tables 1 and 5). Returning to Table 8, maximum resolution for these isomers is generally found for lower T and longer gradient times, but as in the other examples of Table 4, there are exceptions to this rule.

An important qualification to the above generalizations for acidic or basic isomers must be noted. Where the acid or base functional group in the two isomers is equivalent so far as intramolecular electronic or steric effects, e.g., as for the fatty acids that correspond to the esters of Table 5, the pK_a value of

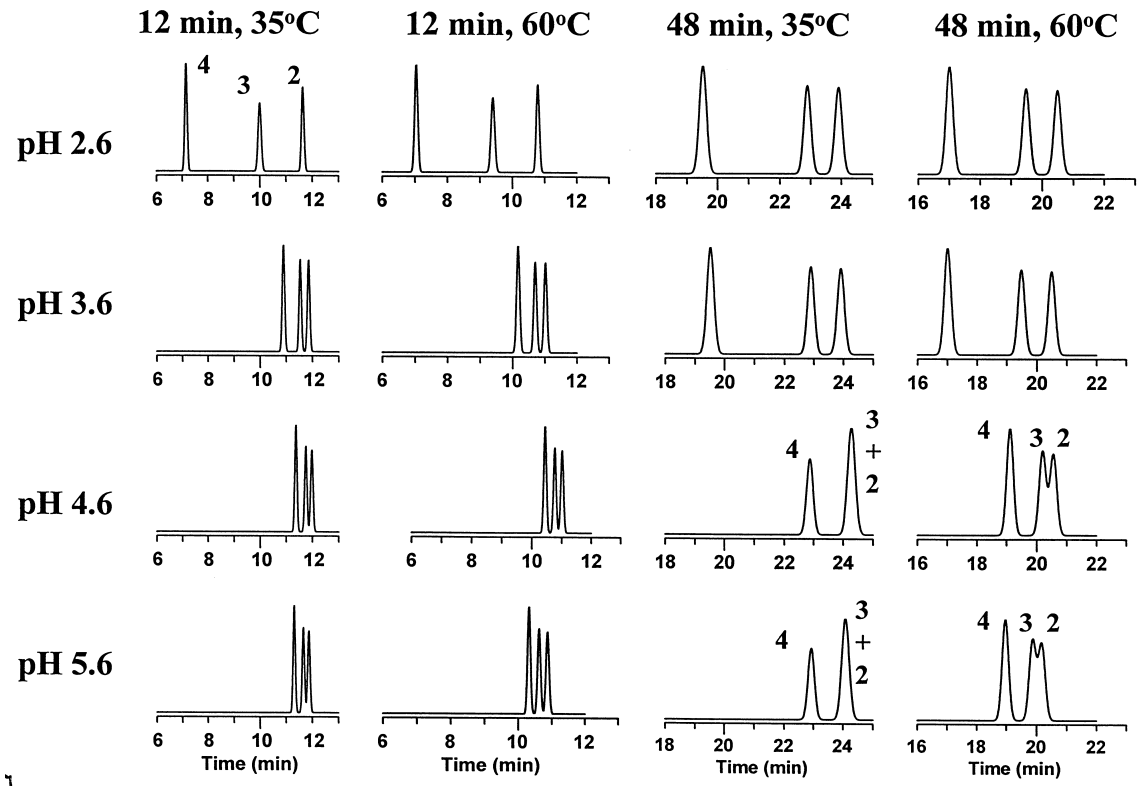

Fig. 5. Separation of 2-, 3- and 4-chloroanilines as a function of pH, gradient time and temperature. Other conditions given in Table 8. The retention sequence is always 4-Cl<3-Cl<2-Cl aniline, except for 48 min, 35°C and pH=4.6 or 5.6, where the 2-Cl and 3-Cl isomers overlap. Separations shown are computer simulations based on the experimental data of Refs. [4,5].

the two isomers will be the same, and α and R_s for the two compounds should then not change with pH. Likewise, values of ΔR_s are also not expected to change with pH. That is, the separation of acid or base isomers of this type by varying T and t_G should be similar to the case of neutral isomers.

Table 9
pK_a values for compounds of Table 7 in 35% methanol in buffer mobile phases at 35°C [17]

Compound	pK_a
2-Fluorobenzoic acid	3.6
3-Fluorobenzoic acid	3.4
2-Chloroaniline	2.1
3-Chloroaniline	2.9
4-Chloroaniline	3.3
3,4-Dichloroaniline	2.3
3,5-Dichloroaniline	2.0

5. Conclusions

The RPLC separation of 137 diverse achiral isomer pairs (37 isomers) has been studied as a function of temperature (T) and gradient time (t_G, equivalent to % B). The effects on separation of column type (monomeric vs. polymeric alkyl-silica), solvent type (acetonitrile vs. methanol) and mobile phase pH was further explored for a smaller number of isomer pairs. On the basis of these results, a number of conclusions can be drawn for this sample set, and presumably for other samples as well. When conditions of T and t_G were optimized, all but 14 (90%) of these isomer pairs could be separated with a resolution $R_s>1.0$, implying values of the separation factor $\alpha>1.05$. Likewise, changes in resolution with T were generally significant, while changes in t_G had an even larger effect on R_s, as

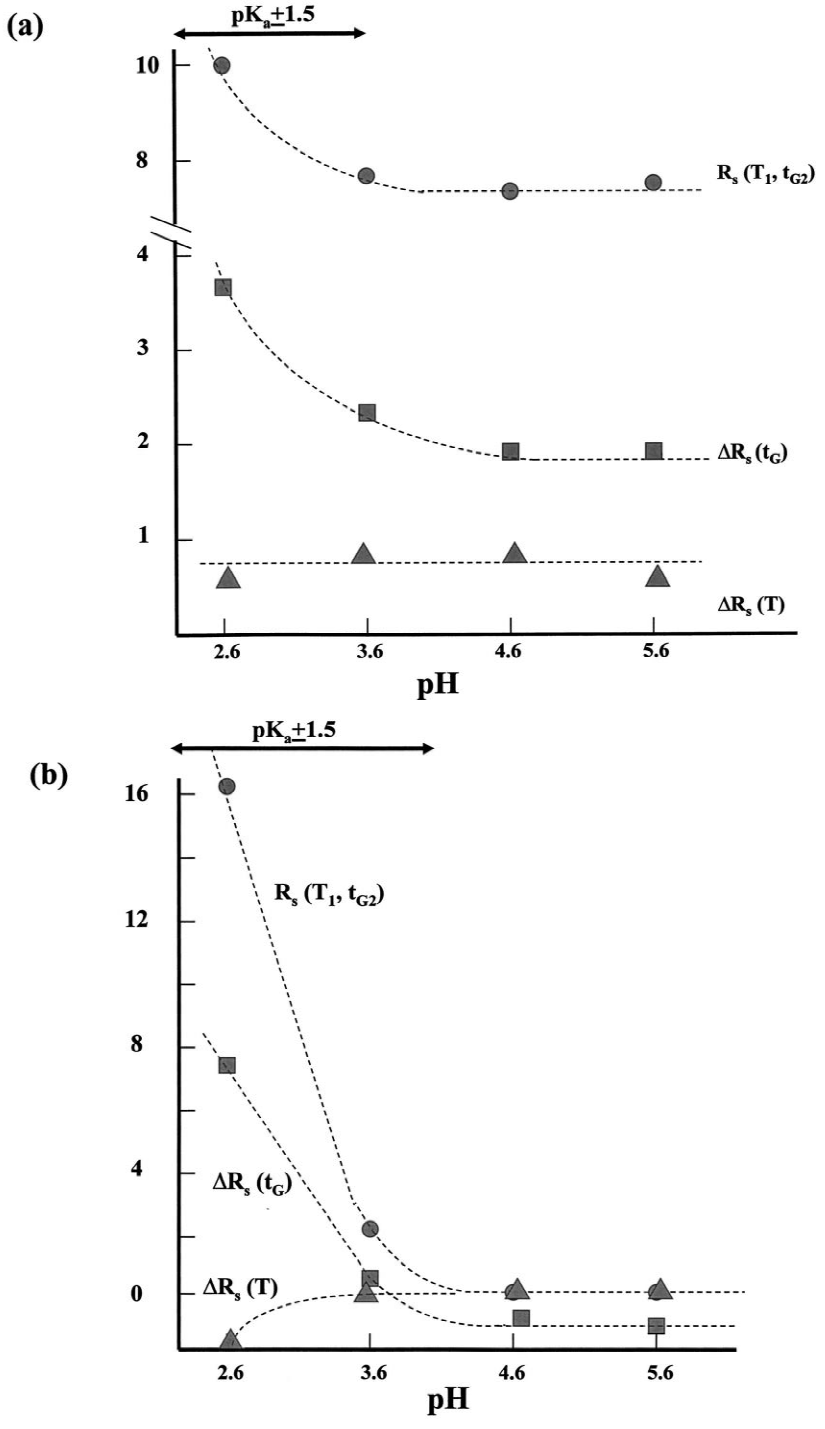

Fig. 6. Resolution and change in resolution of different acids or bases as a function of pH. (a) 3,4- and 3,5-dichloroaniline solutes; other conditions as in Table 7; (b) 2- and 3-chloroaniline solutes; other conditions as in Table 7; (c) 2- and 3-fluorobenzoic acids; other conditions as in Table 7.

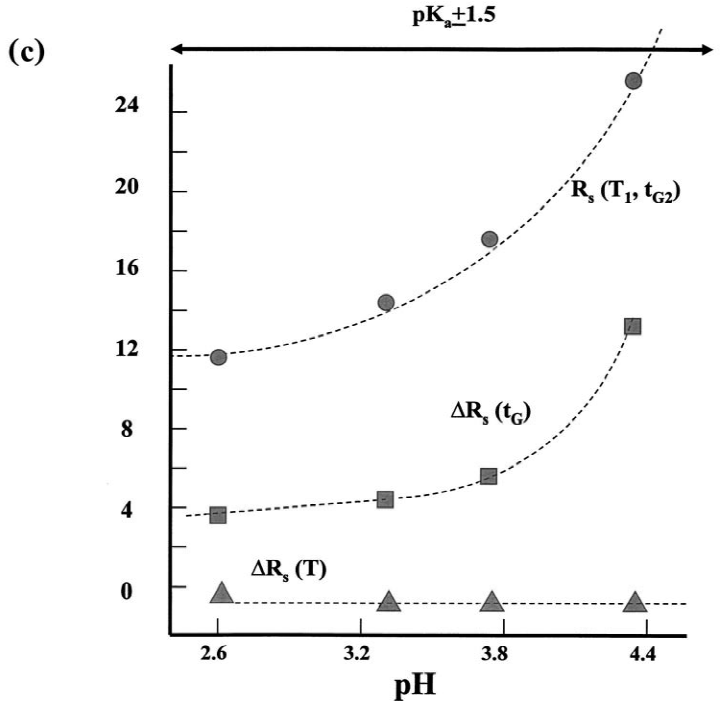

Fig. 6. (*continued*).

observed previously [8] for non-isomeric compounds. Since only four experiments are required to map resolution in RPLC as a function of temperature and either gradient time or isocratic % B, RPLC with the optimization of T and t_G represents a practical approach for the separation of most achiral isomeric samples.

The nature of the sample has a large effect on isomer resolution when T and t_G are optimized. Isomers are more easily separated in the order: nonpolar<polar<partially ionized acids or bases; the effects of T and t_G on resolution or α also increase in the same order. Isomers differing in shape (e.g., *cis* vs. *trans* unsaturated fatty acids, or isomers differing in the position of unsaturation) are better separated on polymeric than on monomeric columns, and the effects of T and t_G on resolution are also greater for polymeric columns. Only minor differences in the resolution of unsaturated fatty-acid-ester isomers were observed when methanol was substituted for acetonitrile, other conditions being the same. However, this may not be the case for other isomers, especially those that are more highly substituted with polar functional groups. There were no significant examples of retention reversal for the present isomers and conditions, over the range in conditions studied.

We believe that the separation of isomer pairs generally will be possible by means of RPLC, especially molecules that are substituted by two or more polar substituents. About 75% of these isomer pairs were best separated using longer gradients (or lower %B) and lower temperatures. Most of the remaining isomer pairs were best resolved with long gradients and higher temperatures (in a few cases, shorter gradients were preferred). However, most samples will contain components in addition to one or more isomer pairs, and the complete separation of such samples will often occur at intermediate conditions of temperature and gradient time.

In the present study, temperature was varied by 25°C, and gradient time was varied by four-fold. Further increase in these two variables (e.g., 40°C and 6-fold in t_G) could easily double the maximum R_s values reported here, suggesting that most of these isomers should be separable with $R_s>1.0$.

6. Nomenclature

F	Means Flow-rate (ml/min)
k	Means Retention factor
R_s	Means Resolution
S	Means solute parameter equal to d(log k)/dφ
t_G	Means Gradient time (min)
t_{G1}, t_{G2}	Means Different gradient times, where $t_{G1} < t_{G2}$
T	Means Temperature (°C)
T_1, T_2	Means Different temperatures, where $T_1 < T_2$
V_m	Means Column dead volume (ml)
α	Means Separation factor
ΔR_s	Means A change in R_s as a result of some change in T (δT) or t_G (δt_G)
$\Delta\varphi$	Means Change in φ during a gradient
φ	Means Volume fraction of B-solvent (e.g., acetonitrile) in the mobile phase

Acknowledgements

The present study was supported in part by a Small Business Innovation Research (SBIR) grant from the National Institutes of Health (US Department of Health and Human Services).

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