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Temperature as a variable in reversed-phase high-performance liquid chromatographic separations of peptide and protein samples
I. Optimizing the separation of a growth hormone tryptic digest

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Abstract

Peptide and protein samples are often complex mixtures that contain a number of individual compounds. The initial HPLC separation of such samples typically results in the poor resolution of one or more band pairs. Various means have been suggested for varying separation selectivity so as to minimize this problem. In this study of a tryptic digest of recombinant human growth hormone, the simultaneous variation of temperature and gradient steepness was found to be a convenient and effective means of varying selectivity and optimizing the separation. The use of computer simulation greatly facilitated this investigation.

1. Introduction

Peptide and protein samples often contain 20 or more individual components. The complete separation of such samples poses a real challenge, because statistical considerations suggest that one or more peak-pairs will usually be poorly resolved [1]. One way out of this dilemma is a systematic variation of separation selectivity. This approach is widely used for the separation of typical small-molecule samples [2]. In the case of peptide and protein samples, however, the control of selectivity has received less attention. When a change in selectivity is desired, the usual approach is a change of the column or mobile phase [3].

The use of elevated temperatures for the reversed-phase HPLC separation of samples containing peptides or proteins has been advocated [4], primarily as a means of increasing column efficiency or shortening run time. For samples of this type, a few studies [5,6] have shown that a change in column temperature can also affect separation selectivity. The most popu-

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lar conditions for the reversed-phase separation of peptide or protein samples involve acetonitrile–water gradients with the addition of 0.1% trifluoroacetic acid (TFA) to maintain a low pH in the mobile phase. However, the combination of low pH (≈ 1.9) and higher temperature operation can result in a very short life for commonly used alkyl-silica columns [7], which in turn limits the application of temperature optimization. This problem has been overcome recently by the development [8] and commercialization of so-called "sterically protected" reversed-phase packings that are extremely stable at low pH ($pH < 2$) and high temperature ($> 90°C$).

For the reversed-phase separation of peptide and protein samples, it has been shown that a change in gradient steepness can be quite effective in changing peak spacing and resolution [9–13]. Because a change in temperature or gradient steepness is more convenient than a change of column or mobile phase, this suggests the *combined* use of temperature and gradient steepness as a means of optimizing the separation of peptide or protein samples. This possibility is explored in the present paper, using a tryptic digest of recombinant human growth hormone (rhGH) as example. In the following paper [14], we examine other samples and consider whether the combined use of changes in temperature and gradient steepness is likely to be generally useful for the separation of peptide or protein mixtures.

2. Theory

2.1. Gradient steepness

The theory of gradient elution is now well established and a good understanding exists concerning the effects of gradient steepness on separation [15–17]. Retention times in gradient elution can be related to sample retention under isocratic conditions, which for reversed-phase separation can be approximated as

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

Here k is the retention factor of the solute, and φ is the volume fraction of organic (acetonitrile) in the mobile phase. Values of k_w and S are characteristic of each solute in the sample. Many examples of the applicability of Eq. 1 for peptide or protein samples have been reported [17–20]. Values of S and k_w for each solute can be obtained from two experimental gradient separations of the sample, and retention times in gradient elution can then be predicted as a function of gradient conditions [17]. Two compounds that elute adjacent to each other in the chromatogram will often show significant changes in band spacing when isocratic solvent strength or gradient steepness is varied [12,21]. The resolution of such band-pairs can usually be accomplished when values of S for the two compounds differ by 5% or more [21].

2.2. Temperature

Small-molecule separations

Much is known concerning the effects of column temperature on reversed-phase separation, primarily from experimental studies of small-molecule samples ($M_r < 1000$). In most cases isocratic retention k can be related to absolute temperature T as

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

A is a function of the phase ratio and entropy of retention, ΔS; B is proportional to the enthalpy of retention (ΔH). Values of ΔH are usually negative, so that retention decreases at higher temperatures. For small-molecule samples, several studies [22–27] have shown that values of $-\Delta H$ and B are usually larger for later bands in the chromatogram. Melander et al. [22] have expressed this relationship quantitatively by noting (for solute molecules of similar structure) that there is often a linear relationship between the enthalpy and entropy of retention. This concept leads to an equation of the form

$$\log k_T = C\Delta H + D \quad (3)$$

Here, k_T refers to k values for different solutes at some temperature T; C and D are

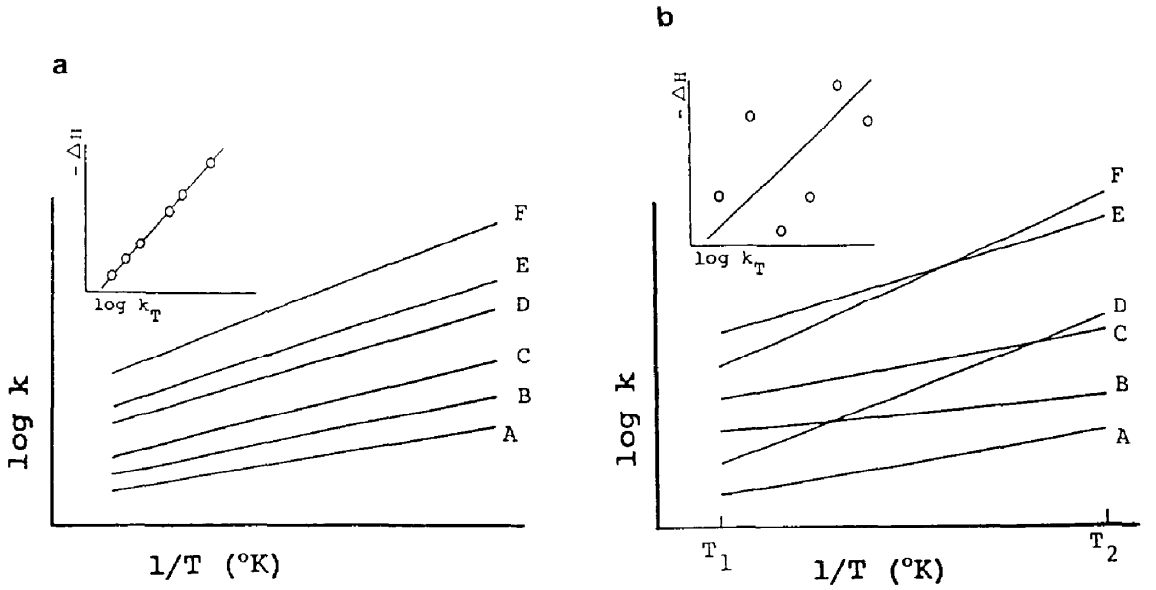

Fig. 1. Hypothetical representation of temperature selectivity for (a) a separation obeying Eq. 3 and (b) a separation deviating from Eq. 3.

constants for separations with the same experimental conditions (including temperature). Fig. 1a illustrates the form of log k vs. $1/T$ plots (different solutes) for a system where Eq. 3 is obeyed.

If Eq. 3 is obeyed exactly for different solutes in a given reversed-phase system, no major changes in selectivity can be expected when the temperature is varied. That is, the sequence of bands in the chromatogram cannot change as a function of temperature, and any bands that are unresolved at one temperature ($\alpha = 1$) will be unresolved at all temperatures. This relationship implies that a change in temperature will be of little value in improving band spacing, which is often the case for the separation of neutral molecules. Fig. 1a illustrates this situation; the retention order of bands A–E (A < B < C < D < E < F) is the same, regardless of the temperature of separation.

While previous workers have generally regarded temperature as a less useful means of controlling selectivity for increased resolution (e.g., [23,25]), a number of small-molecule studies have shown significant changes in band spacing as temperature is varied [27–32]. This failure of Eq. 3 is commonly associated with the existence of more than one separation process or "mechanism", as well as certain related effects: (1) retention of the solute on more than one stationary phase site (e.g., cationic samples retained on alkyl groups vs. silanols); (2) acid–base reactions of the sample molecule with the formation of different species (e.g., AH and A^-, BH^+ and B); (3) other secondary equilibria such as ion pairing. In addition, differences in molecular shape for different solute molecules can lead to a failure of Eq. 3 [33].

Fig. 1b illustrates the consequences when a reversed-phase separation deviates markedly from Eq. 3. The separation order observed at temperature T_2 (A < B < C < D < E < F) is quite different than that observed at temperature T_1 (A < D < B < C < F < E). Band-pairs B/D and E/F are unresolved ($\alpha = 1.0$) at intermediate temperatures, but are well separated at higher and lower temperatures.

A similar pattern is observed for a change in either temperature or φ; if φ replaces $1/T$ in Fig. 1, plots of the same form are obtained. As a result, in isocratic separation run time and *average* resolution decrease for an increase in either

T or φ. However, maximum resolution for the worst-separated band-pair often occurs for intermediate values of T or φ, because of changes in selectivity. For gradient elution, similar changes in selectivity can be effected by (a) a change in gradient steepness (equivalent to a change in isocratic φ) or (b) a change in temperature.

An increase in gradient steepness results in a compression of the chromatogram with narrower bands and a shortening of the run time. A change in temperature for a gradient separation does not result in a compressed chromatogram, due to the elution of each band at a lower value of φ at higher T. That is, the effect of T on k *during* gradient elution is almost exactly cancelled by a corresponding change in φ at elution (this can be seen by comparing Figs. 4 and 5); see also the discussion of Ref. [17].

Separations of peptides and proteins

The above discussion of effects that are likely to lead to deviations from Eq. 3 suggests that changes in selectivity with temperature will often be significant in the separation of peptide and protein samples. These solute molecules usually contain acidic and basic groups whose ionization can vary with pH and temperature. The most common mobile phase (acetonitrile–water plus added TFA) allows ion pairing of basic groups with the TFA [34], and changes in selectivity with temperature are both expected and commonly observed in ion-pair systems. Molecular conformation (and shape) is also known to vary among different peptide and protein solutes. For these various reasons, it should not be surprising if important changes in selectivity are observed for peptide and protein samples as temperature is varied.

The combined effects of a change in temperature T and gradient steepness on the spacing of bands within the chromatogram Δt_R (difference in retention times for two adjacent bands) can be approximated as

$$\Delta t_R = \Delta\Delta t_R(T) + \Delta\Delta t_R(\text{steepness}) \quad (4)$$

where $\Delta\Delta t_R(T)$ refers to a change in Δt_R due to a change in temperature and $\Delta\Delta t_R$(steepness) refers to a change in Δt_R due to a change in gradient steepness. A practical question concerns the relative magnitudes of these temperature and gradient steepness effects and whether they are correlated or independent. If changes in retention of comparable magnitude result from practical changes in T (e.g., by 30–40°C) or gradient steepness (e.g., change in gradient time by a factor of 3–4), either temperature or gradient steepness would be useful for controlling band spacing. If changes in band spacing with temperature or gradient steepness are independent of each other (non-correlated), then the *combined* use of these two variables should be especially useful for controlling selectivity and optimizing separation. On the basis of our present knowledge, there is no obvious reason why any correlation should exist between selectivity effects caused by changes in temperature or gradient steepness.

3. Experimental

3.1. Equipment and materials

The HPLC equipment used is described in Ref. [13]. The column was a 15×0.46 cm Zorbax SB-C_8 (300 Å pore diameter, "sterically protected", 5-μm particles; Rockland Technologies). All experiments used water (A) and acetonitrile (B) gradients, with 0.1% trifluoroacetic acid (TFA) added to each solvent. Other conditions are indicated in the text, tables or figures. Solvents were preheated to the temperature of the column by using a precolumn coil of capillary tubing maintained at column temperature. The tryptic digest of rhGH was prepared as described in Ref. [13].

3.2. Computer simulations

Experimental data were used as a basis for predicting separation as a function of gradient conditions and thereby arriving at general conclusions for the present sample. These predictions were based on computer simulations using DryLab/Windows software (LC Resources, Wal-

nut Creek, CA, USA). The use of this software for similar separations of rhGH tryptic peptides is described in Ref. [13].

4. Results and discussion

4.1. Computer simulation

Experiments were carried out to allow the separation of the rhGH digest to be predicted as a function of gradient steepness and temperature. Three runs each were made at 20, 40 and 60°C (gradient times of 30, 60 and 120 min). The 30- and 120-min runs were used for computer simulation; the 60-min run served as a check on peak tracking and the accuracy of computer simulation. These chromatograms are moderately complex, as seen in Fig. 2 for separation at 60°C with a 120-min gradient. The numbering in Fig. 2 and elsewhere in the present study is the same as was used in Ref. [13]. The numbered peaks represent major peptides, but several smaller peaks are also evident in the chromatogram of Fig. 2. Peak 20 of Fig. 2 is small and often obscured by other minor peaks eluting in this region; for this reason, we ignore peak 20 in further discussions. Our object here is not the optimized separation of this particular sample, but rather the use of these separations to illustrate certain general conclusions.

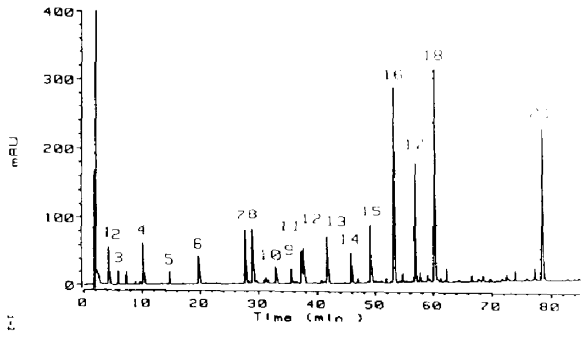

Fig. 2. Separation of 100 μg of rhGH tryptic digest using a 120-min linear gradient at 60°C. Peak numbering as in Ref. [13]. Conditions: column, 15 × 0.46 cm, 300 Å, Zorbax SB-C_8; 0–60% B gradient in 120 min; flow-rate, 1.0 ml/min. Other conditions as in the Experimental section.

The reliability and accuracy of computer simulation was first tested by predicting retention times for the 60-min gradient runs at the three temperatures. These values were compared with experimental retention times as summarized in Table 1. The average error in predicted retention times was ± 0.06 min or 0.3%. Similarly, the average error in predicted retention time differences (proportional to resolution) was only ± 1.1%. These checks assure that predictions based on computer simulation will be sufficiently accurate for our present purpose.

The value of N for these separations was obtained using the present computer simulation software. Either resolution R_s or bandwidth W can be predicted for various values of N. The value of N which gave the best overall agreement between calculated and predicted values of R_s and W was assumed to be correct. Resolution measurements for overlapped peak-pairs gave values of N which ranged from 4800 to 6100, as temperature was varied from 20 to 60° C.

4.2. Effect of changes in gradient steepness and temperature

The DryLab simulation software can summarize resolution as a function of gradient time (steepness) in the form of a resolution map. Examples for the rhGH sample and three different temperatures are shown in Fig. 3, where R_s

Table 1
Verification of computer simulation for the separation of rhGH tryptic digest sample

Temperature (°C)	Average error (min/%)[a]	
	Retention time t_R	Difference in t_R [b]
20	±0.05 min/±0.2%	±0.02 min/±0.9%
40	±0.06 min/±0.3%	±0.02 min/1.0%
60	±0.08 min/=0.4%	±0.03 min/1.3%

Predicted and experimental retention times are compared for the 60-min gradient runs, based on experimental data for 30- and 120-min runs and computer simulation.

[a] Error expressed as either min or %.

[b] Error in retention differences for adjacent bands, proportional to error in predicted resolution.

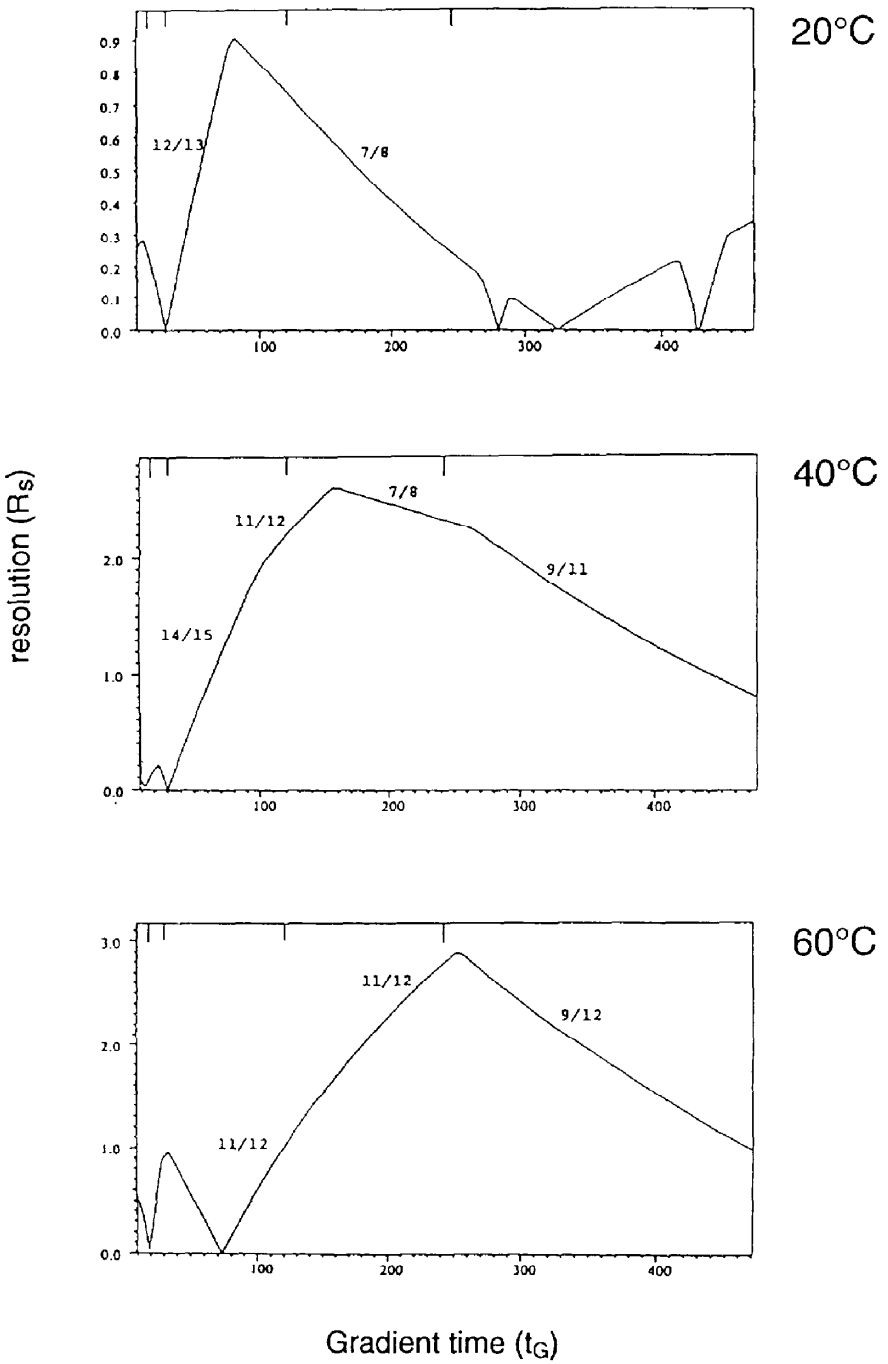

Fig. 3. Resolution maps of rhGH tryptic digest as a function of gradient time and temperature (20, 40 and 60°C). Other conditions as in Fig. 1. Numbers refer to critical (least resolved) band-pairs.

for the critical (least resolved) band-pair is plotted vs. gradient time. If temperature were unimportant as a determinant of selectivity and peak spacing, these three maps would look generally similar, with little change in the maximum attainable resolution. As seen in Fig. 3, this is not the case. Each resolution map has a quite different appearance; the optimum gradient time varies from 70 min for separation at 20° to 250 min at 60°, and the maximum attainable resolution varies from $R_s = 0.9$ (20°C, poor) to $R_s = 2.8$ (60°C, excellent). The critical peak-pairs (numbers shown within Fig. 3) also change with temperature (see discussion below).

The above observations concerning Fig. 3 suggest that temperature is a potentially important variable for controlling band spacing and resolution in the case of peptide mixtures. Thus the combined variation of temperature and gradient steepness as in Fig. 3 allows a much greater sample resolution than can be attained via changes in gradient time alone. This is also evident in the change in critical band pairs as temperature is varied. Peak-pairs 7/8 and 12/13 are least resolved at 20°C, peak-pairs 7/8, 9/11, 11/12 and 14/15 are critical at 40°C, and peak-pairs 9/12 and 11/12 limit resolution at 60°C.

The effect of gradient steepness on controlling peak spacing and resolution is illustrated in Fig. 4, where the critical peaks 7–15 are shown for three different gradient times (30, 60 and 120 min) and a temperature of 40°C. Peak-pair 7/8 is little affected by change in gradient time, whereas peak-pairs 9/10, 11/12 and 14/15 become better resolved as gradient time increases. A similar comparison of the effect of temperature on the separation of these bands is shown in Fig. 5. Here peak-pair 7/8 becomes better separated as temperature increases, peak-pair 11/12 is best separated at an intermediate temperature (40°C), and peak-pair 14/15 reverses between 20 and 60°C. The advantage of a good choice of gradient steepness and temperature is apparent in the examples of Figs. 4 and 5. The best of these separations (Figs. 4 and 5) is seen for a temperature of 40°C and a gradient time of 120 min; see also the resolution maps of Fig. 3.

4.3. Prediction of separation as a function of temperature and gradient steepness: solute retention parameters S and k_w for each peptide

Computer simulation for the prediction of the reversed-phase gradient separation of peptide or protein samples has been described [10–13]. Two initial experimental separations are used to derive values of the isocratic parameters S and k_w for each sample component (Eq. 1). Table 2 summarizes values of S (software derived) for the various peptides of the rhGH digest. Data for S as a function of temperature have been reported for several small-molecule samples (see review of Ref. [35]). Generally, there is no change or a slight decrease in S with increasing temperature. This trend was also observed for the rhGH digest as shown in Fig. 6. Here, values of S at 20°C are plotted vs. values at 60°C. The solid line for $y = x$ in Fig. 6 confirms that S is relatively independent of temperature for this peptide sample, considering likely errors in the derived values of S. Consequently, the effects of a change in gradient steepness on peak spacing should be similar at different temperatures.

Values of the solute parameter log k_w also vary with temperature, as summarized in Table 3. The quantity k_w is defined as the value of k for an isocratic separation with 0% B (no organic). Values of log k_w generally decrease with temperature, as expected for a retention process that releases heat when the solute molecule is sorbed onto the column packing. If values of S and k_w are interpolated from the data of Tables 2 and 3 as a function of temperature, retention times and resolution can be predicted for any temperature and gradient conditions. This approach would allow the generation of resolution maps as in Fig. 3 for other temperatures, so as to define the best possible values of gradient time and temperature for this sample. Further work is required to confirm that this simple approach will be applicable (a reviewer has suggested that the scatter of data in Fig. 6 could represent a possible complication).

The selection of "best" values of temperature and gradient steepness for an HPLC separation

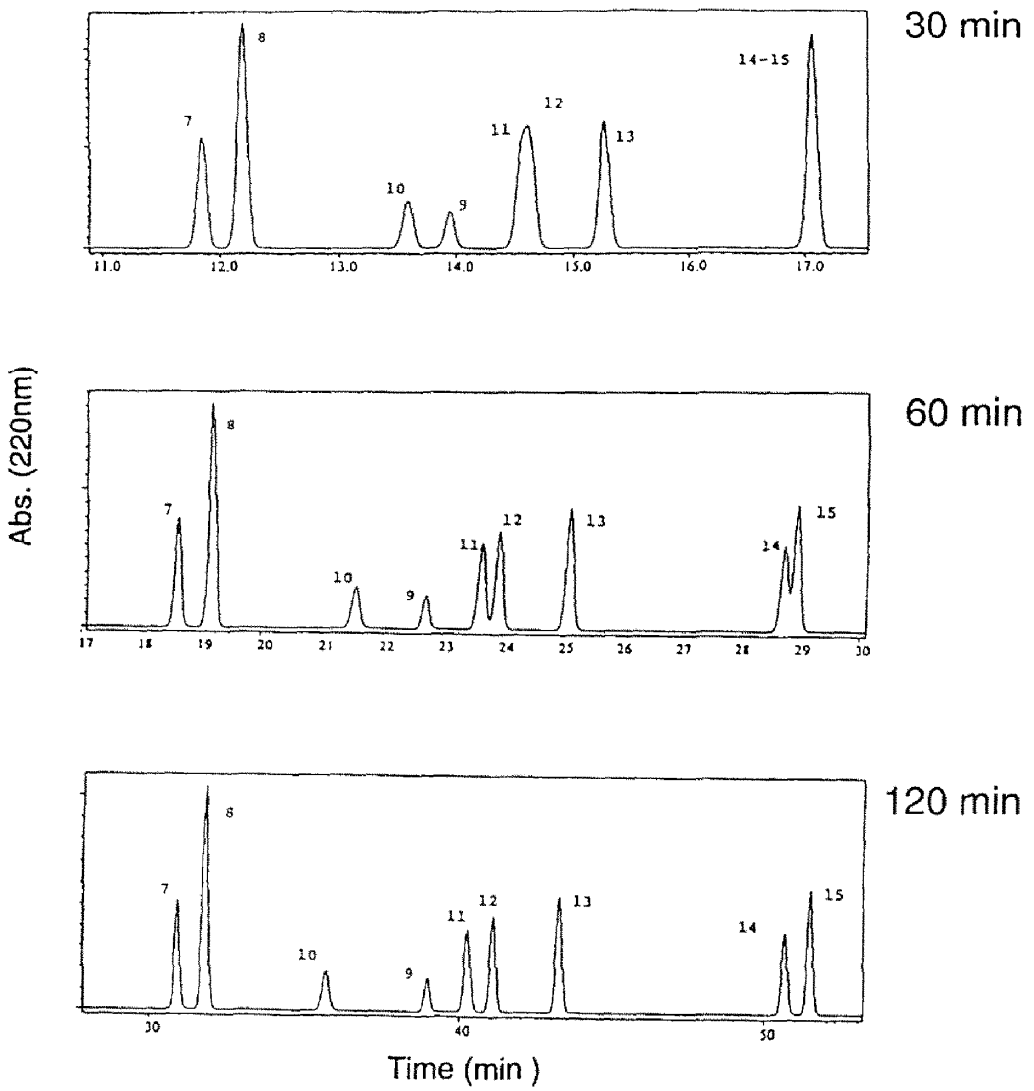

Fig. 4. Separation of rhGH tryptic peptides 7–15 as a function of gradient time (steepness). Conditions as in Fig. 1, 40°C.

requires consideration of the relative importance of both resolution and run time. It is convenient to choose an adequate resolution (e.g., baseline separation or $R_s > 1.5$) and then select the shortest possible run time. From the data of Fig. 2, $R_s > 1.5$ can be achieved with an 85-min gradient at 40°C or a 150-min gradient at 60°C, but not at all at 20°C. Thus, in this case, a temperature of 40°C is better than either 20 or 60°C.

4.4. Non-correlation of selectivity effects due to changes in temperature or gradient steepness

Figs. 3–5 for the rhGH digest suggest that selectivity effects due to a change in temperature are different from effects due to a change in gradient steepness. That is, the simultaneous variation of temperature and gradient steepness should be especially useful in optimizing sepa-

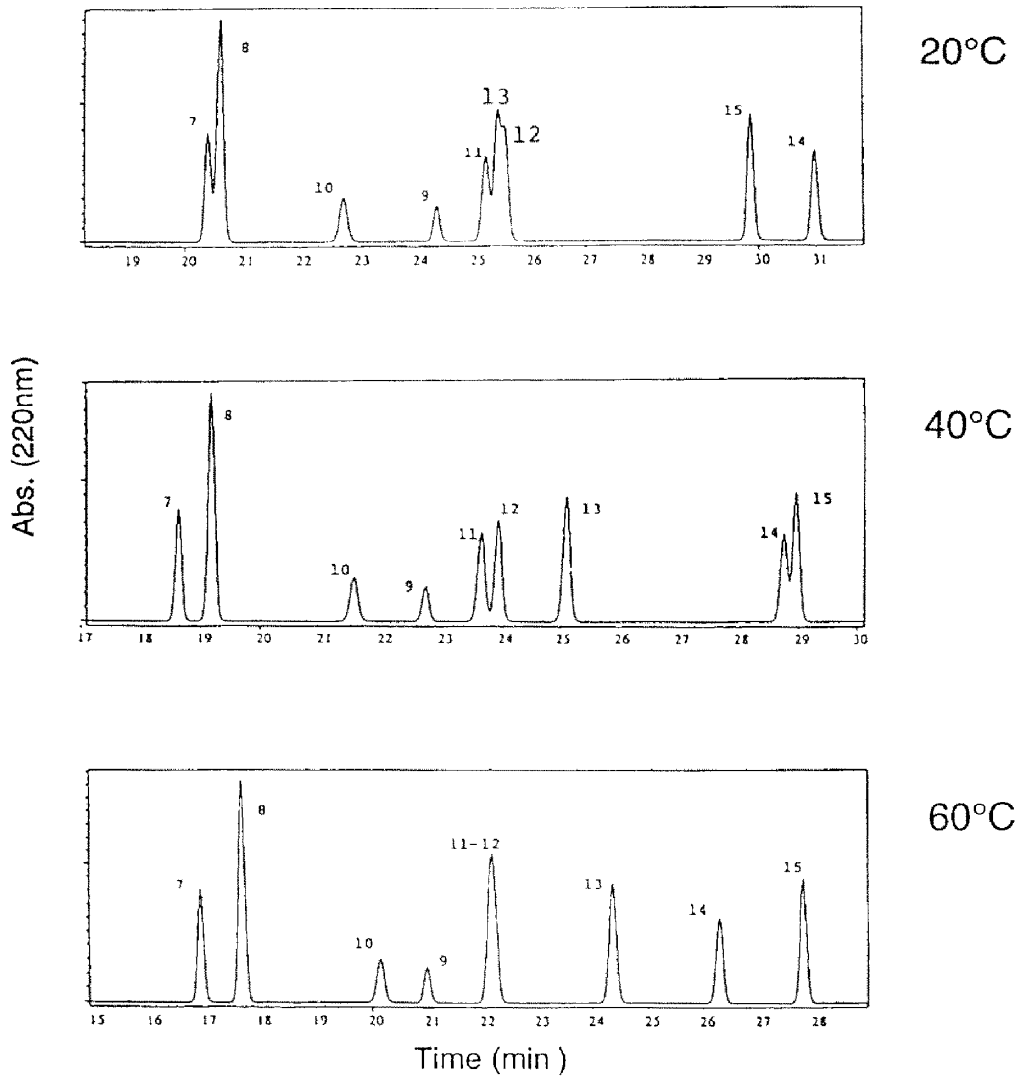

Fig. 5. Separation of rhGH peptides 7–15 as a function of temperature. Conditions as in Fig. 1, 60-min gradients.

ration. A more objective statement of this conclusion can be reached as follows. Table 4 summarizes values of $\Delta t_R(T)$ for a change in temperature from 60 to 20°C, corrected for differences in gradient time. Average values of $\Delta t_R(T)$ are reported for each peptide. Differences in $\Delta t_R(T)$ for each pair of adjacent peptides [equal to $\Delta\Delta t_R(T)$] are also reported. The quantity $\Delta\Delta t_R(T)$ is a direct measure of a change in selectivity due to temperature; values of $\Delta\Delta t_R > 0.5$ min are indicative of band-pairs whose resolution can be affected significantly by a change in temperature. The spacing of band-pairs 7/8 ($\Delta\Delta t_R = -0.6$), 9/10 (-0.7), 13/14 (3.0) and 14/15 (-2.2) should be responsive to a change in temperature, and this is illustrated in the examples of Fig. 5.

The temperature-selectivity data of Table 4

Table 2
Values of the gradient-steepness parameter S for the rhGH peptides

Peak	S at each temperature				Difference in S for adjacent bands (ΔS)
	20°C	40°C	60°C	Average	
2	24.3	31.1	22.0	(26)[a]	
3	15.7	18.5	23.6	(19)[a]	
4	23.7	26.6	28.5	(26)[a]	
5	12.5	13.6	14.1	13.4	1.5
6	14.2	15.1	15.5	14.9	9.0
7	22.8	24.2	24.8	23.9	−2.0
8	21.3	22.1	22.3	21.9	0.6
9	22.3	22.6	22.5	22.5	−5.9
10	16.8	16.7	16.4	16.6	2.4
11	17.4	18.7	21.0	19.0	0.9
12	21.7	20.3	17.7	19.9	−0.9
13	19.8	19.0	18.1	19.0	1.1
14	20.7	19.8	19.9	20.1	2.5
15	22.7	22.9	22.2	22.6	1.4
16	27.6	28.3	28.1	28.0	−3.1
17	24.9	25.0	24.4	24.9	8.9
18	32.7	34.7	34.0	33.8	−2.6
19	30.7	31.9	31.1	31.2	−12.9
21	18.5	18.5	17.9	18.3	

Determined from data for 30- and 120-min gradient times.
[a] Less reliable values due to early elution of bands.

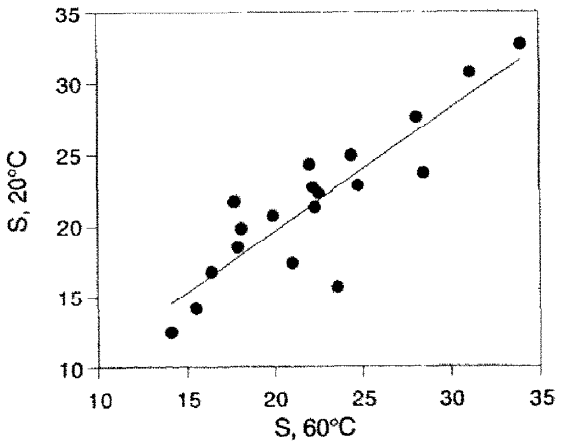

Fig. 6. rhGH tryptic peptide values for S at 20°C vs. 60°C (software derived, see Table 2).

plus S values from Table 2 allow a test of whether simultaneous changes in temperature and gradient steepness are worthwhile for the present sample. If values of $\Delta t_R(T)$ do *not* correlate with values of S, then it can be concluded that the effects of temperature and gradient steepness operate independently, and it will be profitable to change temperature and gradient selectivity simultaneously. If, on the other hand, a strong correlation exists between $\Delta t_R(T)$ and S, the use of either temperature or gradient steepness should result in similar selectivity effects; i.e., the simultaneous use of these two variables would be less useful.

Fig. 7 compares changes in band spacing as a result of changes in temperature and gradient steepness. Values of Δt_R for a change in temperature from 60 to 20°C (average values from Table 4), are plotted vs. average values of S (Table 2) for peptides 5–21 in the rhGH sample. The

Table 3
Values of the gradient-steepness parameter k_u for the rhGH peptides

Peak	Log k_u at each temperature		
	20°C	40°C	60°C
2	0.90	0.59	0.24
3	1.07	0.79	0.52
4	1.74	1.49	1.18
5	1.62	1.50	1.32
6	2.11	1.99	1.77
7	3.87	3.64	3.31
8	3.71	3.50	3.20
9	3.40	3.19	2.91
10	4.68	4.36	3.96
11	3.92	3.89	3.97
12	4.43	4.23	3.46
13	4.83	4.22	3.92
14	6.01	5.10	4.02
15	5.75	5.85	5.43
16	7.85	7.67	7.22
17	7.36	7.24	6.81
18	10.00	10.33	9.78
19	9.66	9.84	9.33
21	7.46	7.50	7.18

Determined from data for 30- and 120-min gradient times.

marked scatter of the resulting plot ($r^2 = 0.00$) indicates no correlation of these two quantities, confirming the value of simultaneous variation of temperature and gradient steepness for the purposes of controlling band spacing.

4.5. Column stability

The use of temperatures >50°C for the low-pH reversed-phase separation of peptide or protein samples has been avoided in the past because of the instability of available bonded-phase columns. The use of low-pH mobile phases (e.g., 0.1% TFA in acetonitrile–water) can lead to a rapid loss of bonded phase, even at temperatures near ambient [36–39]. This loss in bonded phase causes continuous changes in retention time and a loss in column plate number. Such changes complicate peak identity assignments, make quantitation more difficult, and require more frequent (and expensive) column replacement.

Recent improvements in the bonded phases available for reversed-phase HPLC have resulted in commercially available columns that are much more stable toward low-pH, high-temperature operation [8,40,41]. These new columns (StableBond, Rockland Technologies) are based on "sterically protected" silanes, in which the methyl groups of the usual alkyldimethylsilane bonded phase are replaced with bulkier groups such as isopropyl or isobutyl. The use of these monomeric sterically protected phases ensures repeatable separations and excellent column performance during the life of the column, even for aggressive separation conditions. This in turn makes possible the effective use of temperature optimization as illustrated in the present study.

5. Conclusions

Narrow- and wide-pore "sterically protected" reversed-phase column packings are now commercially available for use under low-pH, high-temperature conditions. These silica-based packings allow temperatures as high as 90°C (without loss of bonded phase) for the efficient separation of peptide or protein samples. This in turn makes possible the use of temperature as a means of varying separation selectivity. In the present study of the separation of a tryptic digest of rhGH, it was found that peak spacing changed significantly when column temperature was varied from 20 to 60°C. It was further observed that the combined use of temperature and gradient steepness provided an efficient procedure for the control of peak spacing and optimization of separation for this sample. At the same time, this approach to selectivity control is more convenient than alternatives such as a change of column or mobile phase, because temperature and gradient steepness can be varied via the system controller. The following paper [14] provides further examples of this kind, which suggests that this approach to the optimized separation of peptide and protein samples is generally applicable.

Table 4
Changes in retention time t_R for a change in temperature from 60 to 20°C as a function of gradient time t_G

Band	Change in t_R (min) for different t_G [a]				Inter-band change ($\Delta\Delta t_R$)[b]
	30 min	60 min	120 min	Average	
2	3.9	3.6	2.2	3.0	0.6
3	4.4	3.6	2.7	3.6	−0.7
4	3.2	2.9	2.6	2.9	0.1
5	3.2	2.8	2.5	2.8	0.2
6	3.2	3.0	2.8	3.0	0.5
7	3.6	3.4	3.4	3.5	−0.6
8	3.0	2.9	2.8	2.9	0.4
9	3.4	3.3	3.3	3.3	−0.7
10	2.5	2.6	2.6	2.6	0.5
11	3.4	3.0	2.8	3.1	0.1
12	3.0	3.2	3.4	3.2	−0.2
13	0.9	1.2	1.5	1.2	3.4
14	4.6	4.7	(3.3)	4.6	−2.5
15	2.0	2.0	2.1	2.1	0.7
16	2.8	2.7	2.8	2.8	−1.1
17	1.6	1.7	1.7	1.7	0.0
18	1.8	1.7	1.7	1.7	−0.3
19	1.4	1.3	1.4	1.4	−1.1
21	0.3	0.4	0.3	0.3	

[a] Values for 30- and 120-min runs are corrected for the effect of gradient time; data for 30-min runs have been multiplied by 2 and the data for the 120-min runs have been multiplied by 1/2.
[b] E.g., for bands 6/7, it is the average change for band 7 minus the change for band 6; i.e., $\Delta\Delta t_R = 3.5 - 3.0 = 0.5$.

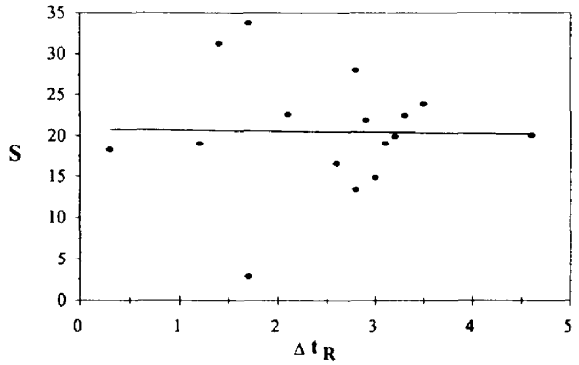

Fig. 7. Non-correlation of temperature and gradient-steepness selectivity effects for the rhGH sample. The change in peptide retention time (Δt_R, min) for a change in temperature from 60 to 20°C (average values of Table 4) is plotted vs. the average value of S (Table 2) for bands 5–21. $\Delta t_R = 20.8 - 0.13S$; $r^2 = 0.0003$; error y (S) estimate = 7.5.

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