ELSEVIER

Journal of Chromatography A, 686 (1994) 45–59

JOURNAL OF CHROMATOGRAPHY A

Temperature as a variable in reversed-phase high-performance liquid chromatographic separations of peptide and protein samples
II. Selectivity effects observed in the separation of several peptide and protein mixtures

Rosanne C. Chloupek[a], William S. Hancock[a,1], Brian A. Marchylo[b], Joseph J. Kirkland[c], Barry E. Boyes[c], Lloyd R. Snyder[d,*]

[a]*Genentech, Inc., 460 Point San Bruno Boulevard, South San Francisco, CA 94080, USA*
[b]*Canadian Grain Commission, Grain Research Laboratory, Winnipeg, Manitoba, Canada*
[c]*Rockland Technologies Inc., 538 First State Boulevard, Newport, DE 19804, USA*
[d]*LC Resources Inc., 2930 Camino Diablo, Suite 110, Walnut Creek, CA 94596, USA*

First received 31 December 1993; revised manuscript received 2 August 1994

Abstract

Changes in band spacing as a function of temperature and/or gradient steepness were investigated for four peptide or protein samples. Reversed-phase HPLC in a gradient mode was used to separate tryptic digests of tissue plasminogen activator and calmodulin. Additionally, a synthetic peptide mixture and a storage protein sample from wheat were studied. Simultaneous changes in gradient steepness and temperature were found to provide considerable control over band spacing and sample resolution.

The effects of temperature and gradient steepness on selectivity in these systems appear to be complementary. Simultaneous optimization of both temperature and gradient steepness thus represents a powerful and convenient means of controlling band spacing and separation. Because of the complexity of these sample chromatograms, computer simulation proved to be a useful tool in both interpreting these experiments and in optimizing final separations.

1. Introduction

The preceding paper [1] noted possible problems in achieving the separation of complex peptide or protein samples. One approach is to change conditions so as to optimize selectivity or peak spacing within the chromatogram. Several established means of altering separation selectivity have been reported [2]: column source or type (e.g., C_4 vs. cyano), change in the organic solvent (acetonitrile, propanol, etc.), the use of ion-pairing conditions, variation of pH or ionic strength. Nevertheless, most reversed-phase separations of peptide and protein samples em-

* Corresponding author.
[1] Present address: Hewlett-Packard Laboratories, 3500 Deer Creek Road 26U, Palo Alto, CA 94304, USA.

0021-9673/94/$07.00
SSDI 0021-9673(94)00778-O

ploy a "standard" set of preferred conditions: a short-chain alkyl-silica column (e.g., C_3 or C_4) and gradient elution with an acetonitrile–water mobile phase containing ca. 0.1% trifluoroacetic acid (TFA). While the use of these conditions has several practical advantages, one consequence is that practical workers make only limited use of changes in conditions in order to control peak spacing and separation.

The preceding paper [1] suggests that the advantages of low-pH acetonitrile gradients can be retained while selectivity is varied by means of simultaneous changes in temperature and gradient steepness. In this paper we examine whether this approach for optimizing the separation of peptide and protein samples is likely to prove widely applicable.

2. Experimental

2.1. Equipment, materials and procedures

The equipment used to collect the experimental data reported here is described in Refs. 3–7. The columns were 15 × 0.46 cm Zorbax, 300 Å pore diameter, 5-μm SB-C_8 for recombinant tissue plasminogen activator (rt-PA) and the cereal storage protein sample. The calmodulin and synthetic peptide separations used a 15 × 0.46 cm Zorbax, 80 Å pore, 3.5-μm SB-C_{18} column. Each of these columns (Rockland Technologies) has a "sterically protected" bonded phase that is stable at low pH and high temperature.

The materials and samples used in the present study are described in Refs. 3 (cereal proteins), 5 (calmodulin digest) and 7 [reduced and S-carboxymethylated (RCM) rt-PA digest].

All gradients used water (A) and acetonitrile (B) with 0.1% added TFA.

Computer simulations were carried out as described in the preceding paper [1], using DryLab/windows software (LC Resources).

2.2. Tryptic mapping (LC–MS)

A 100-μl aliquot containing 100 μg of the RCM rt-PA tryptic digest was loaded onto a 15 × 0.46 cm Zorbax SB-C_8 column at a flow-rate of 0.5 ml/min using a Hewlett-Packard 1090M HPLC system. The sample was separated with 0–60% B gradients in 30 and 120 min. Column temperature was 40°C. The effluent was split 1:20 with a Valco tee, to give a flow-rate of 25 μl/min into the ionspray nebulizer (Sciex). The balance of the column effluent was diverted to the HPLC system (monitored at 220 nm).

MS analysis was carried out in quadrupole —one of a Sciex API III triple-quadrupole mass spectrometer. The quadrupole was scanned from 300 to 2000 u in 4.3 s, using a step size of 0.5 u and a 1.2-ms dwell time per step. The intensity of the plot was adjusted manually to minimize interference due to background.

3. Results and discussion

3.1. RCM rt-PA tryptic digest

Computer simulation

Initial runs at 30, 60 and 120 min for temperatures of 20, 40 and 60°C were carried out as in the case of the recombinant human growth hormone (rhGH) digest [1]. As expected for a digest with 54 theoretical peptide fragments, the resulting chromatograms were much more complex and poorly resolved (Fig. 1a). In addition to the major peptides (labeled in Figs. 2–5; see Ref. [7] for structures), there are a large number of minor peaks. Because of the complexity of these chromatograms, peak tracking was quite difficult. The use of area measurements for this purpose was complicated by the frequent overlap of minor peaks with peptides of interest and changes in relative peak position as a function of temperature and gradient steepness. This difficulty was largely overcome by two expedients. First, chromatograms measured at 280 nm were used in selected cases, since these chromatograms were less complicated and somewhat easier to interpret. Additionally, area ratios for 280 nm vs. 220 nm detection provided a further check on peak matching. Second, on-line MS measurements were carried out on the 30- and 120-min runs at 40°C, which aided both matching and identification of the major peptide peaks in these runs.

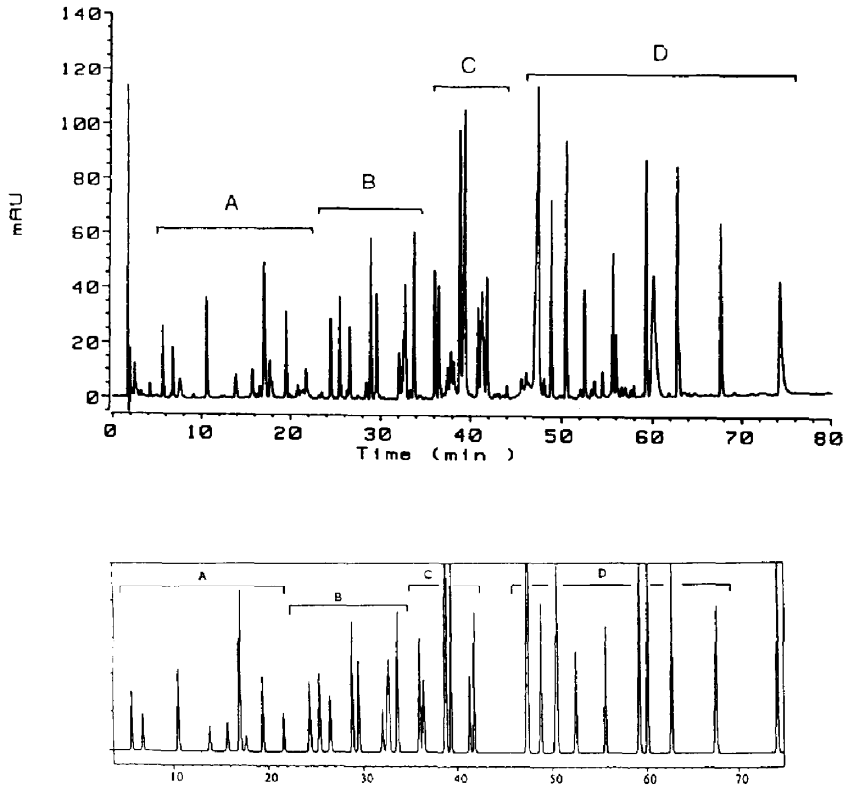

Fig. 1. Separation of rt-PA tryptic digest at 40°C with a 0–60% B 120-min gradient. Other conditions as in Fig. 2 of Ref. [1]. (Top) Experimental chromatogram; (bottom) Computer simulation for major peptides only. Letters A–D designate groups of peptides discussed in text.

Data for the 60- and 120-min runs at a given temperature were used as input to computer simulation, with comparison of experimental and predicted retention times for the 30-min run. The results were similar to those reported in Ref. [1] for the rhGH sample; peak matching and subsequent computer simulations appeared to be accurate and reliable. The use of input runs (60- and 120-min gradients) that differed in gradient time by only a factor of 2 was expected to decrease the accuracy of measured gradient steepness (S) values. However, very crowded chromatograms made direct use of the 30-min gradient data for computer simulation inconvenient and potentially less reliable. Experimental values of the column plate number N for these various separations were determined from resolution measurements as described in Ref. [1]: $N = 4400$ (20°C), 5600 (40°C) and 7000 (60°C).

Effect of changes in gradient steepness and temperature

While rt-PA digest data were obtained for three different temperatures, only the 40 and 60°C data were used for computer simulation. The lower value of N for the 20°C runs combined with the large number of bands to make it almost impossible to untangle the major peptide bands for peak tracking and data entry. Another complication was the complete overlap of certain band-pairs for all values of gradient steepness at 20, 40 or 60°C. This made the use of total-sample resolution maps (as in Fig. 3 of Ref. [1]) unfeasible, because at each temperature one or more critical band pairs had $R_s = 0$ for all values of gradient time t_G. Resulting maps would therefore be completely uniformative. For these and other reasons, we divided the chromatogram into four separate groups of bands (A–D) as shown

in Fig. 1 (lower panel). We will illustrate the effects of temperature and gradient steepness for these individual groups (as in Ref. [1] for the rhGH sample).

Fig. 2 shows chromatograms of group A at 40 and 60°C for both 60- and 120-min gradients. Peaks X1-A, -B and -C comprise peptides T9, T36 and T44, but it was not possible to determine which band corresponds to a specific peptide using only the 40°C MS identification. An increase in temperature improves the separation of the group of peptides labeled "X1" and "T39"; these compounds are optimally resolved at 60°C with a 60-min gradient.

Fig. 3 shows chromatograms of group B as a function of temperature and gradient steepness. This group of compounds exhibits striking changes in relative band position as both temperature and gradient steepness are varied. Group B would be adequately separated ($R_s > 1.0$) with a temperature of 40°C and a gradient time of 90 min (not shown).

Fig. 4 shows chromatograms of group C as a function of temperature and gradient steepness.

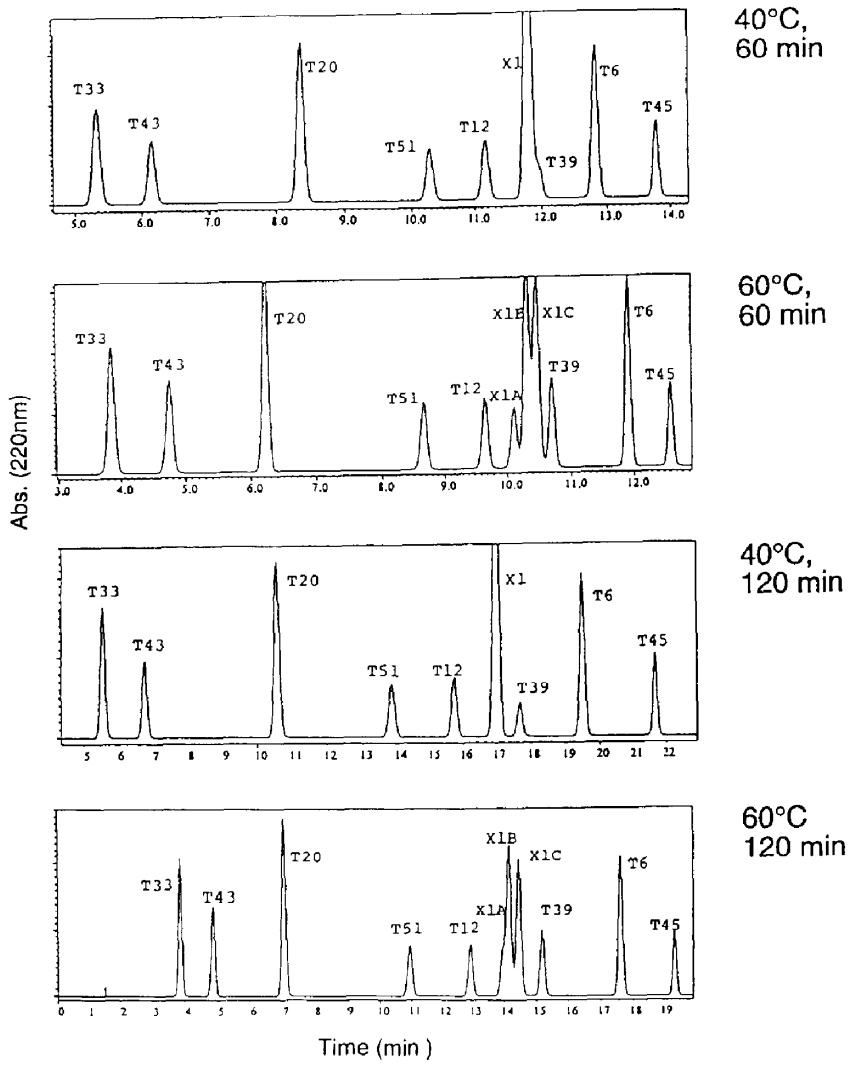

Fig. 2. Effect of changes in temperature and gradient steepness on the separation of group A of the rt-PA digest (see Fig. 1). Temperature and gradient times indicated next to each panel. Computer simulations for N = experimental value.

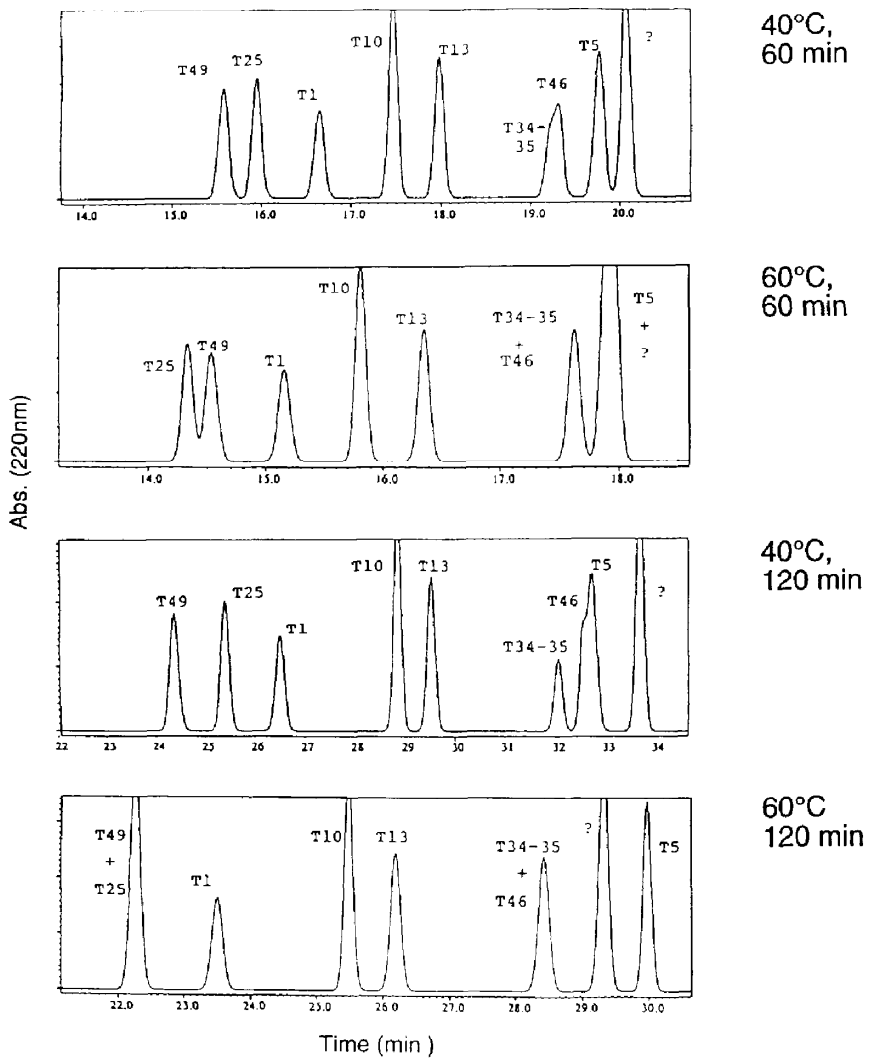

Fig. 3. Effect of changes in temperature and gradient steepness on the separation of group B of the rt-PA digest (see Fig. 1). Temperature and gradient times indicated next to each panel. Computer simulations for N = experimental value.

Again, large changes in relative band position occur as a function of varying temperature or gradient steepness. Complete separation of this group is predicted for a 120-min gradient and a temperature of about 50°C (not shown).

Fig. 5 shows chromatograms of group D as a function of temperature and gradient steepness. Early bands X3-A, -B and -C (comprising T15, T24 and an unidentified peptide) are only separated at 60°C and a gradient time of 120 min. A good separation of this group should result for a gradient time of 120 min and a temperature of about 50°C (not shown).

The examples of Figs. 2–5 further confirm that a variation of temperature is often a powerful means for changing the band spacing of a peptide sample. Not only can major changes in band position be effected by a change in temperature, but these effects appear to be independent of concomitant changes that result from changes in gradient steepness. This is further shown in Fig. 6, where the change in retention with tempera-

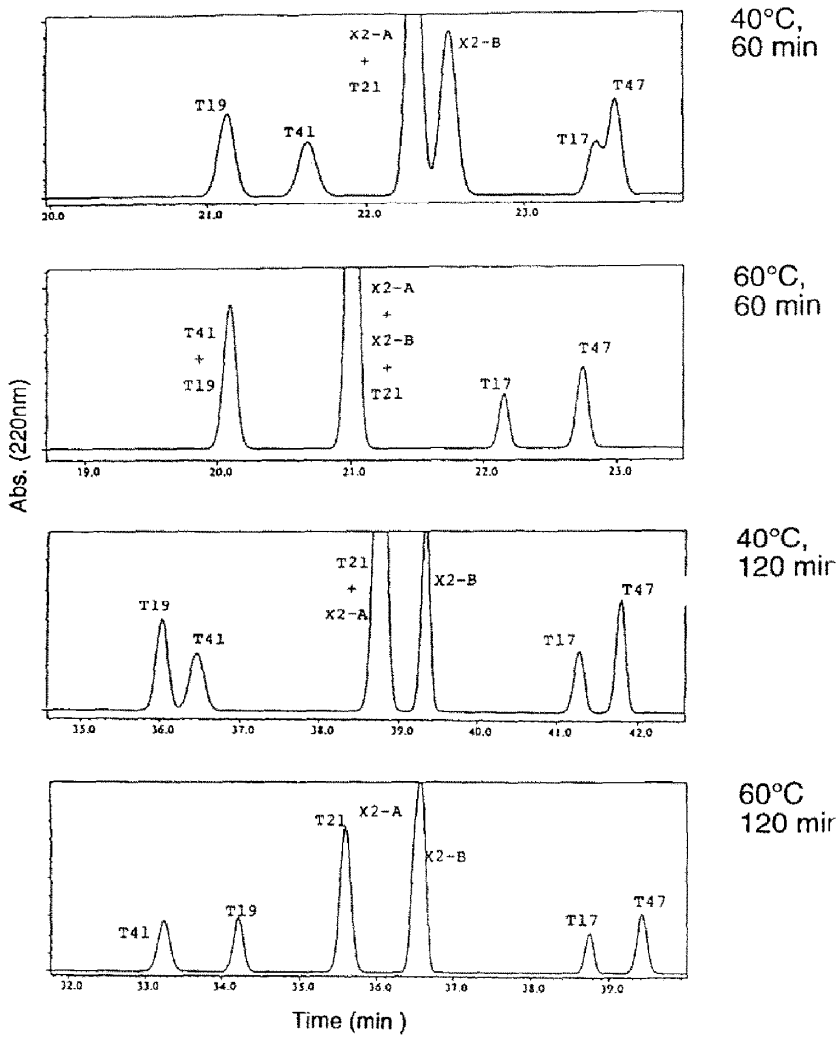

Fig. 4. Effect of changes in temperature and gradient steepness on the separation of group C of the rt-PA digest (see Fig. 1). Temperature and gradient times indicated next to each panel. Computer simulations for N = experimental value.

ture (Δt_R) is plotted vs. S for the various peak-pairs of the rt-PA digest; cf. discussion of Fig. 7 of Ref. [1]; the correlation ($r^2 = 0.10$) is quite low.

As in the case of the rhGH sample [1], values of S do not change much with temperature. This is illustrated in Fig. 7, where values of S for 40°C are plotted vs. values for 60°C.

3.2. Calmodulin tryptic digest

This sample was separated at two different temperatures (35 and 85°C) as shown in Fig. 8. The resulting chromatograms are much simpler than seen previously, with only a dozen or so major peaks being separated. The 85°C separation exhibits narrower bands (as expected), but apart from this difference the two chromatograms look quite similar. This suggests that little change in peak spacing has resulted as a result of this large change in temperature. Upon closer inspection, however, there are at least three significant changes in selectivity. The resolution of doublet "A" in Fig. 8 is the same at the two temperatures, despite the decrease in bandwidth at 85°C. This means that the retention time

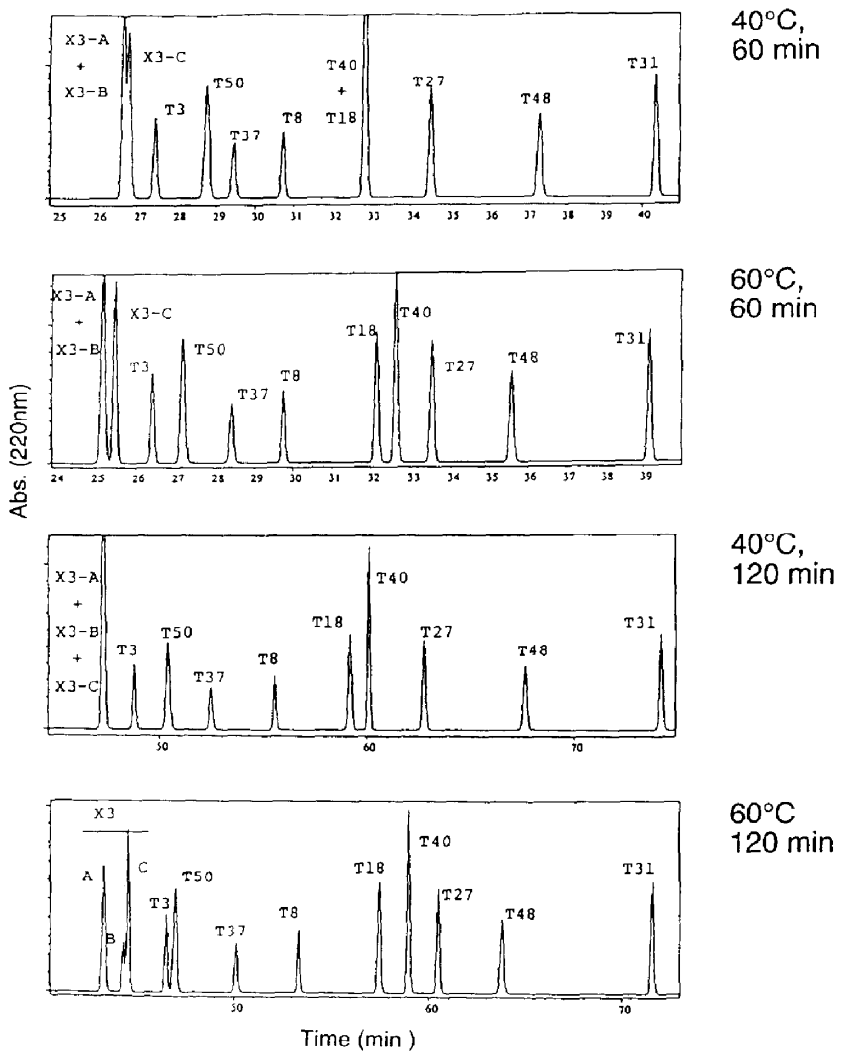

Fig. 5. Effect of changes in temperature and gradient steepness on the separation of group D of the rt-PA digest (see Fig. 1). Temperature and gradient times indicated next to each panel. Computer simulations for N = experimental value.

difference for this peak-pair is actually reduced somewhat as temperature increases. A similar (but more pronounced) decrease in retention time difference is seen for peak-pair B as temperature is increased. Finally, peak-pair C is unseparated at 35°C but is completely resolved at 85°C.

The best overall separation of the calmodulin digest probably occurs at 85°C. The effects of gradient steepness on this separation were not studied, but it is likely that a further improvement in resolution could have been effected by optimizing gradient time as well as temperature.

3.3. *Cereal protein sample*

The separation of this sample is shown in Fig. 9 for two different temperatures: 50 and 70°C. Chromatograms were actually obtained for 30, 50 and 70°C and for gradient times of 30, 60 and 120 min. Because of the complexity of this sample, it was not feasible to carry out computer simulations for the entire sample. Nevertheless, the data obtained so far allow us to examine the utility of temperature variation as a means of controlling band spacing and selectivity for this sample.

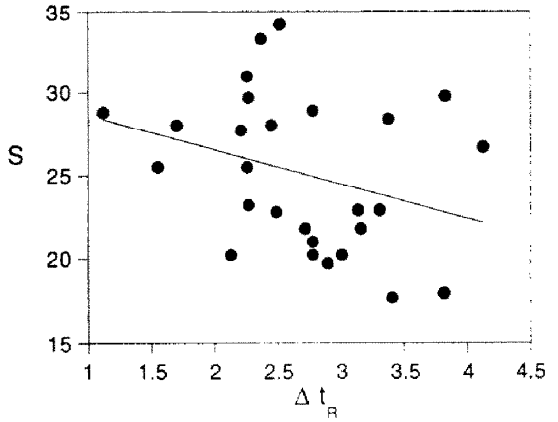

Fig. 6. Non-correlation of temperature and gradient-steepness (S) selectivity effects for the rt-PA tryptic digest sample. The change in peptide retention time (Δt_R, min) for a change in temperature from 60 to 40°C (120-min gradient) is plotted vs. the average value of S for the peak-pair. $\Delta t_R = 3.92 - 0.050S$; $r^2 = 0.105$; standard error y (Δt_R) estimate = 0.65.

Three different groups of compounds in this sample are identified in Fig. 9: peaks A–F, G–J and K–P. We were able to carry out provisional peak tracking for each of these three groups, which allowed subsequent computer simulations as for the rt-PA digest sample. A best fit of the simulated and experimental chromatograms yielded a plate number of $N = 1000$ for the 50°C separations and $N = 1500$ for the 70°C runs. The predicted values (Ref. [8]) for an average column and compounds of this molecular mass were $N = 1600$ and 2250, respectively. The lower experimental plate numbers (by about 35%) suggest a minor problem with either (a) column efficiency or (b) "non-ideal" effects which are common for protein samples [9]. As expected, the use of a higher temperature (70°C) results in a significant increase (20%) in peak capacity. However, this effect is subordinated to changes in selectivity with temperature. The chromatograms for 30°C separation were markedly less resolved than for 50 or 70°C. This is probably the result of both a lower plate number and more serious "non-ideal" effects at 30°C.

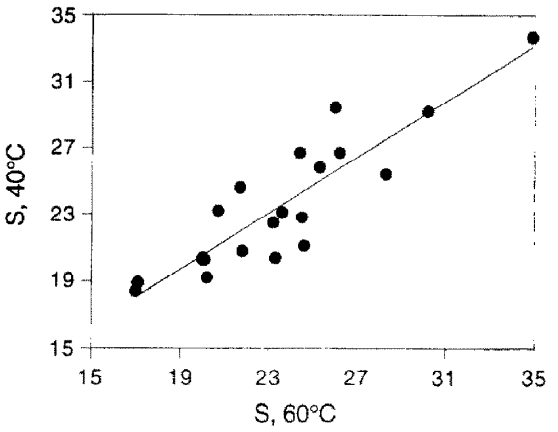

Fig. 7. rt-PA tryptic peptide values for S at 40 vs. 60°C (software derived).

Computer simulations were carried out for 50 and 70°C separations as a function of gradient time. Input values for 60- and 120-min runs allowed the prediction of retention times for the 30-min gradients, as a check on the reliability of peak tracking and computer simulation. Experimental and predicted retention times at 30°C were in agreement within $< 1\%$.

A qualitative comparison of the two separations of Fig. 9 shows several apparent changes in band spacing due to this difference in temperature. For example, the two band-clusters indicated by arrows are markedly different in appearance. At 50°C three major bands plus a large shoulder are apparent, while at 70°C only one distinct band plus two shoulders can be seen. It should also be kept in mind that the higher peak capacity at 70°C would otherwise favor the better separation of this group of proteins.

Fig. 10 shows the separation of group A–F as a function of temperature and gradient steepness. No changes in retention order were noted for the major peaks in this group, although there are obvious changes in selectivity as a function of temperature. Peaks D and E move apart as temperature is increased, while peak C moves from B toward D at the higher temperature. On balance, the best separation of this group is obtained at 50°C with a gradient time of 120 min.

Fig. 11 shows the separation of the largest peaks in group G–J. While these peaks are well resolved for all conditions, we see that peak I moves closer to band J as temperature is increased. The separation of group K–P (Fig. 12) shows that peaks L/M and O/P are only resolved at 70°C with a 120-min gradient. The best

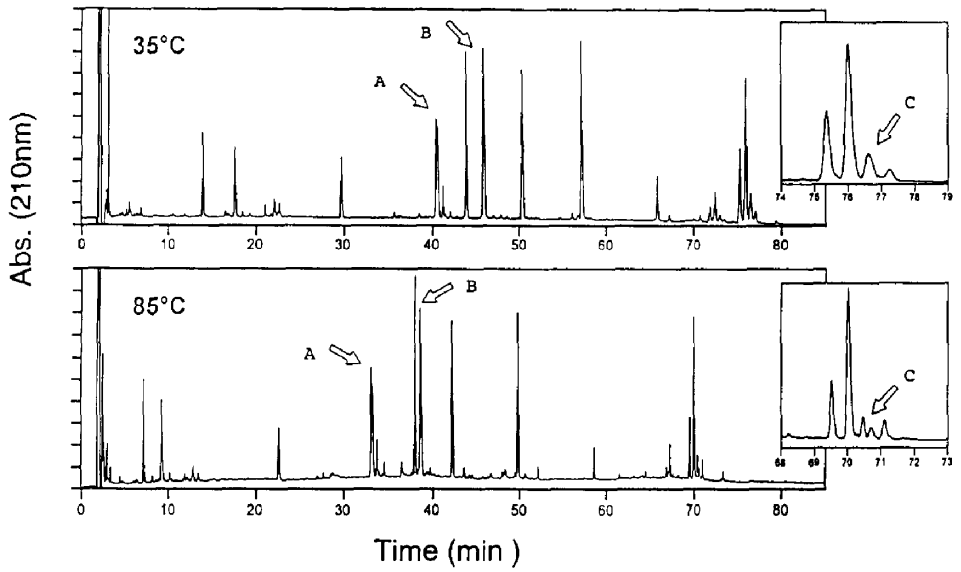

Fig. 8. Calmodulin tryptic digest. Separation at two different temperatures. Conditions: 15 × 0.46 cm Zorbax 80 SB-C_{18} column (3.5 μm); solvent A, 0.1% TFA in water; solvent B, 0.1% TFA in acetonitrile; gradient of 5–60% B in 90 min; 0.75 ml/min; detection at 210 nm. See Ref. [6] for details.

overall separation of this group of bands is obtained at 70°C with a 120-min gradient.

As for the case of the rt-PA sample, values of S for bands A–P were compared at 50 and 70°C (Fig. 13). Somewhat greater scatter was noted vs. the correlation of Fig. 7, presumably because of the greater complexity of these chromatograms and resulting errors in retention time measurements. The correlation of changes in retention time (50°C value minus 70°C value, 120-min gradients) with average values of S (Fig. 14) was somewhat better than for the rhGH and rt-PA samples: $S = 108 - 10.6\Delta t_R$, $r^2 = 0.50$. However the standard error of S (14.1) is large enough to confirm that the temperature selectivity effects observed for the cereal protein sample are both significant and relatively independent of changes in selectivity as a result of change in gradient steepness.

3.4. Synthetic peptide mixture

A sample of five synthetic peptides described by Mant and Hodges [10] was separated by reversed-phase gradient elution at three temperatures (35, 60 and 85°C), other conditions the same; see Fig. 15 and Table 1. These peptides (labeled A–E) are structurally related decapeptides which are acetylated at the amino terminus and amidated at the carboxyl terminus (except for A which is unacetylated). The only other structural difference is in the amino acids at positions 3 and 4 from the amino terminus: A and C, Ala–Gly; B, Gly–Gly; D, Val–Gly; E, Val–Val. Because of these small differences in peptide structure, smaller temperature selectivity effects were expected.

Fig. 15 (and Table 1) show that bands A and B move apart as temperature is increased. Due to the wider spacing of bands B–E, changes in their relative retention are visually less apparent. Table 1 summarizes the changes in retention time with temperature for each peptide; it is seen that the increase in retention time as temperature is decreased from 85 to 35°C decreases regularly from band A to E. Also, a reduced spacing of *all* bands in the chromatogram results as temperature decreases, which results in an increase in resolution of all bands in this sample at the higher temperature, apart from any change in column efficiency with temperature.

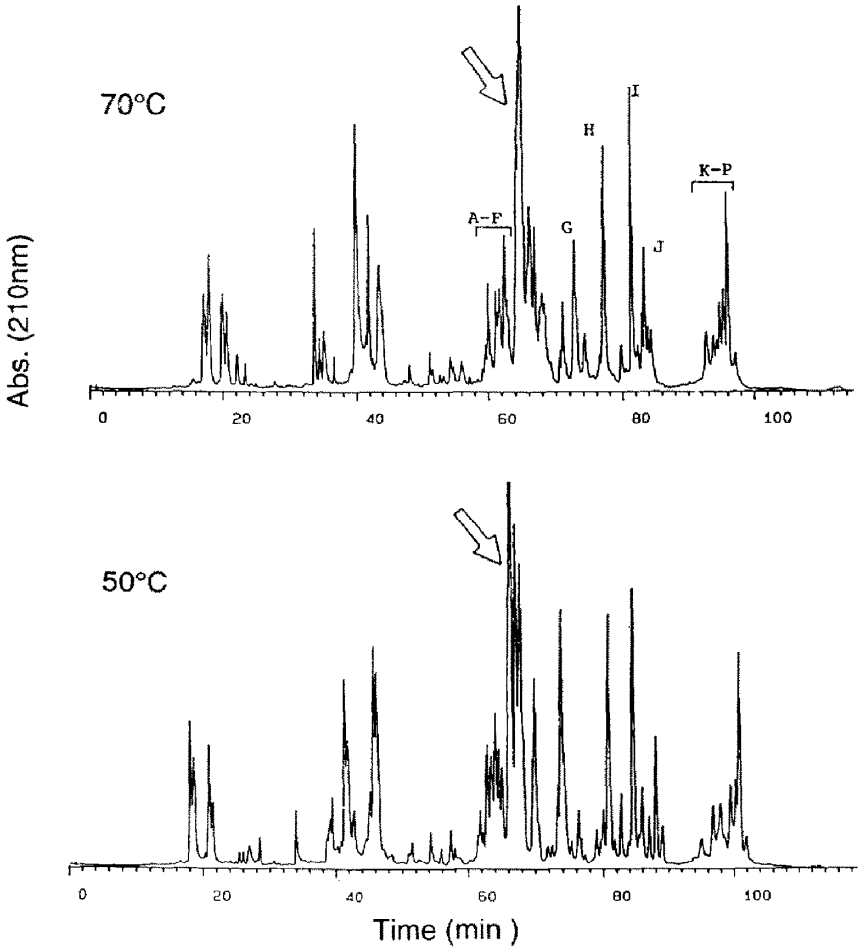

Fig. 9. Separations of cereal storage proteins as a function of temperature. Conditions: 15 × 0.46 cm Zorbax Rx-300 C_8, 23–48% B gradient (acetonitrile–water plus 0.1% TFA) in 120 min, 1 ml/min, detection at 210 nm. Temperature as noted in figure.

Finally, impurity I of Fig. 15 moves towards peak E as temperature increases, so that the separation of all six bands (A–E plus I) is optimum for a temperature between 60 and 85°C.

Curiously, bandwidth *increases* with increasing temperature in the separations of Fig. 15. This is in contrast to the other samples described here and in Ref. [1]. While the experimental bandwidths of Table 1 for a temperature of 35°C are close to values predicted for a well-packed column, the experimental values at 85°C are about 40% higher than predicted.

3.5. The practical application of temperature/gradient optimization

The foregoing examples suggest that band spacing for the reversed-phase separation of peptide or protein samples can be effectively manipulated by the simultaneous variation of gradient steepness and temperature. The application of this approach to different samples will take different forms, however, depending on sample complexity. We can differentiate arbitrarily between samples containing more or less than 25 major components (the same sample

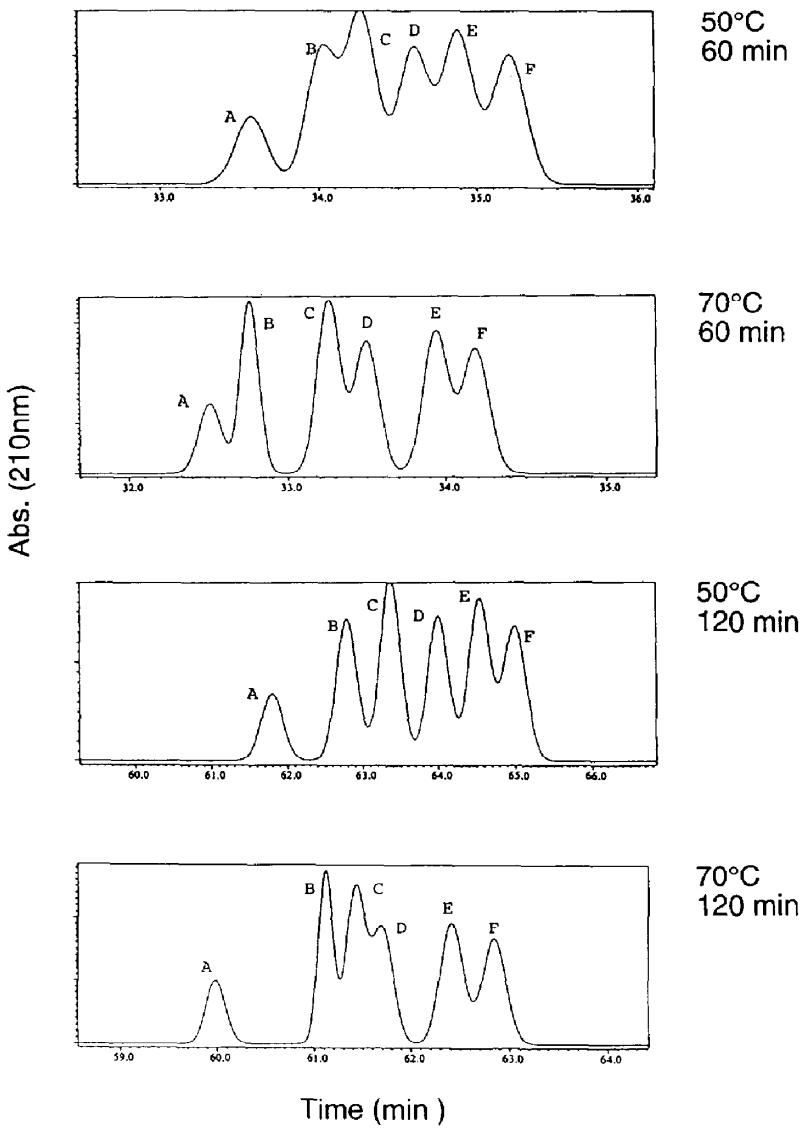

Fig. 10. Separation of cereal protein peaks A–F of Fig. 9 as a function of gradient steepness and temperature. Conditions as in Fig. 9 except as noted. Computer simulations for N = experimental value.

may be assigned to either group, depending on which components we define as "major").

Less-complex samples (<25 major components)

The expectation is that samples of this type will be separable by some combination of gradient conditions and temperature. Initial runs should be carried out at a temperatures which will maximize column efficiency; e.g., 40–80°C. Computer simulation can then yield resolution maps for different temperatures, as in Fig. 3 of Ref. [1]. If the best choice of temperature and gradient time appears unpromising, it may be necessary to explore other means of changing

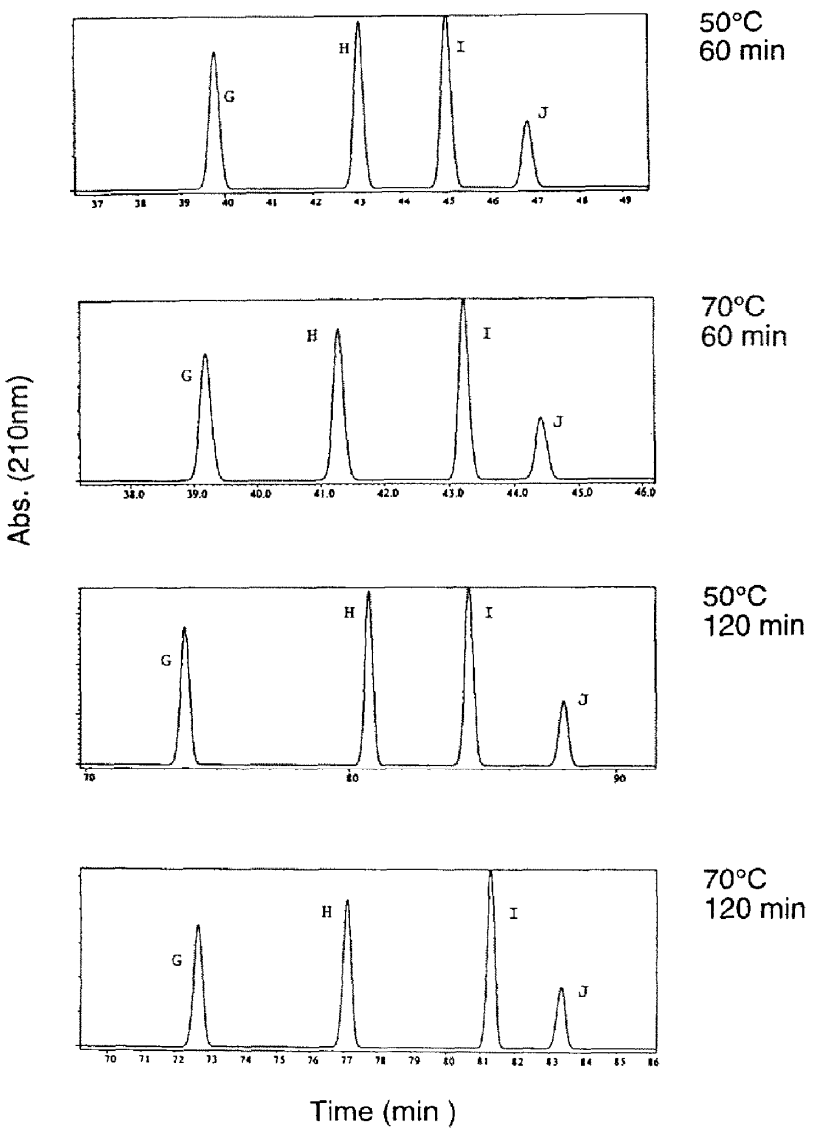

Fig. 11. Separation of cereal protein peaks G–J of Fig. 9 as a function of gradient steepness and temperature. Conditions as in Fig. 9 except as noted. Computer simulations for N = experimental value.

selectivity [2]. Alternatively, the use of a longer, smaller-particle column may prove successful.

More-complex samples (> 25 major components).

As shown in the present examples of the rt-PA digest and cereal protein samples, it is unlikely that a single HPLC separation will be able to resolve all the bands in samples such as this —regardless of gradient and temperature optimization. Very complex samples usually require an initial separation into a small number of fractions by one HPLC method (e.g., ion exchange), followed by further separation of each fraction with a different HPLC method (e.g., reversed phase).

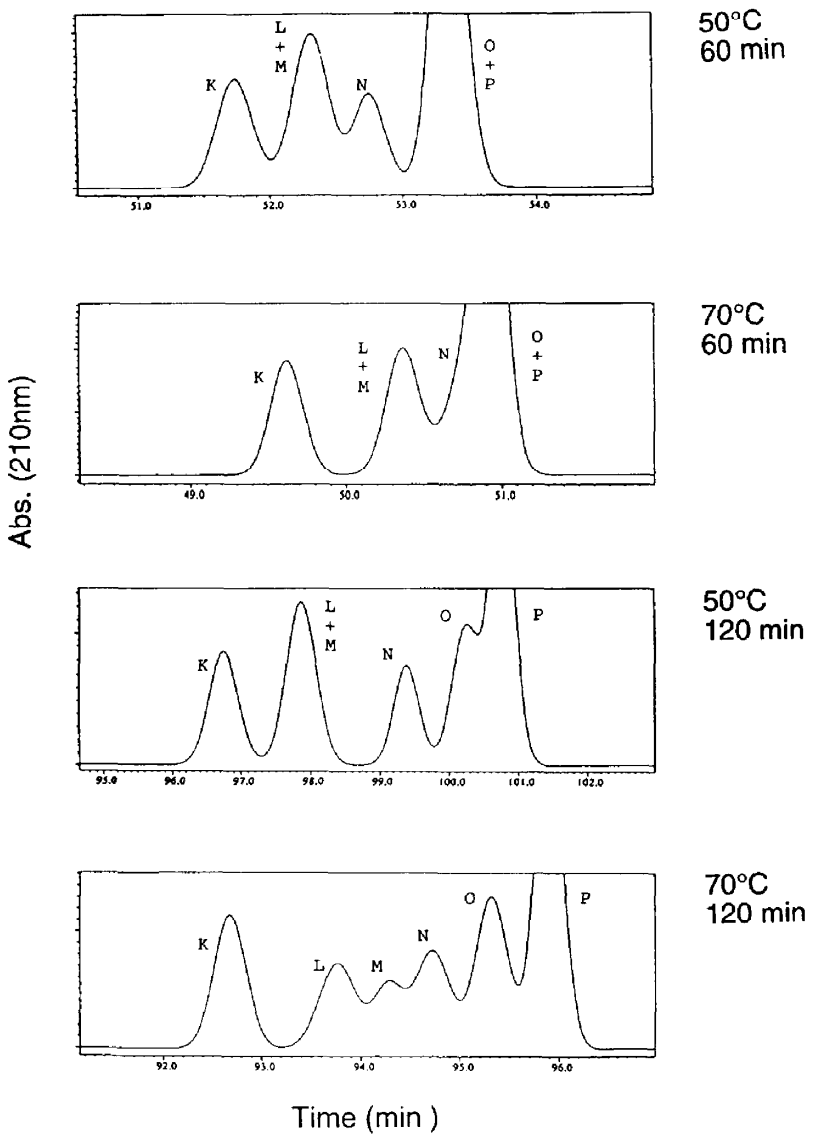

Fig. 12. Separation of cereal protein peaks K–P of Fig. 9 as a function of gradient steepness and temperature. Conditions as in Fig. 9 except as noted. Computer simulations for N = experimental value.Cereal protein sample.

An alternative is to optimize temperature and gradient steepness for the adequate separation of different sub-sets of the sample. This would have been successful in the case of the rt-PA digest, where each of the sub-groups A–D were separable by some choice of temperature and gradient time.

4. Conclusions

Separations by reversed-phase gradient elution of three peptide and one protein sample were studied as a function of temperature and/or gradient steepness. As in the case of the rhGH peptide sample of Ref. [1], it was found that

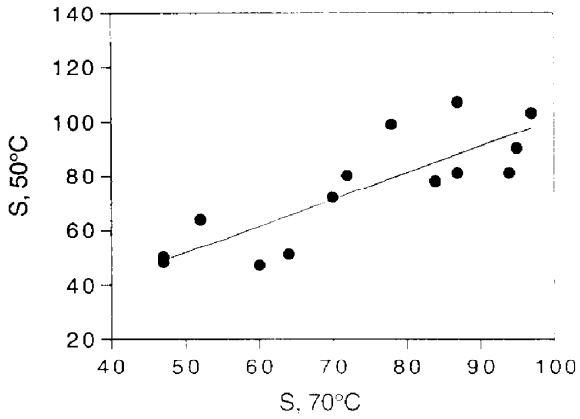

Fig. 13. Cereal storage protein values for S at 40 vs. 60°C (software derived).

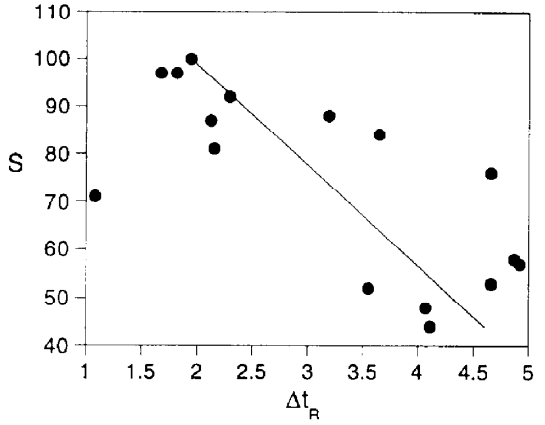

Fig. 14. Non-correlation of temperature and gradient-steepness (S) selectivity effects for the cereal storage protein sample. The change in protein retention time (Δt_R, min) for a change in temperature from 70 to 50°C (120-min gradient) is plotted vs. the average value of S for the peak-pair.

these two variables each have a considerable effect on the spacing of bands within the chromatogram. Their combined effects cannot be duplicated by varying temperature while holding gradient steepness constant nor by varying gradient steepness while holding temperature constant. There should therefore be a considerable advantage in optimizing temperature and gradient steepness simultaneously for the separation of other peptide and protein samples. The convenience of controlling separation by simple adjustments in temperature and gradient steepness represents an additional advantage of this approach. One limitation of this approach is that the use of low-pH mobile phases requires columns that are stable under these conditions; e.g., sterically protected C_8 or C_{18} columns.

For very complex mixtures which contain a large number of both major and minor components, no realistic change in selectivity will

Table 1
Summary of retention times and bandwidths for the separation of the synthetic peptide sample (Fig. 15) as a function of temperature

Peptide	Retention time (min)			
	35°C	60°C	85°C	Δt_R [a]
A	9.73	9.04	7.97	1.76
B	9.84	9.22	8.25	1.59
C	10.28	9.71	8.78	1.50
D	11.60	11.08	10.22	1.38
E	13.11	12.72	11.97	1.14
Average bandwidth[b]				
Experimental	6.3 s	6.6 s	7.1 s	
Calculated	6.7 s	5.7 s	5.0 s	

[a] Difference in retention time, 35°C minus 85°C; e.g., the difference in retention times (Δt_R) for band A is 9.73 − 7.97 = 1.76 min.
[b] Baseline bandwidth values; experimental values average for five peptides; calculated values according to Ref. [9] ($A = 0.5$).

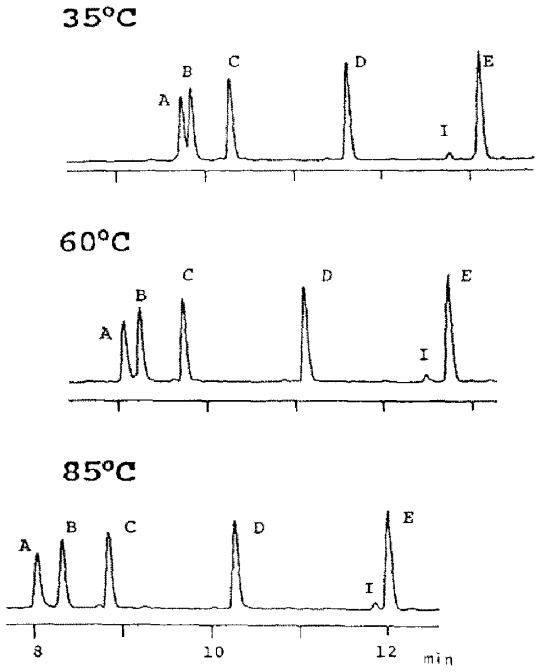

Fig. 15. Synthetic peptide sample. Separation at different temperatures. Conditions: column, 15 × 0.46 cm Zorbax SB-C_{18} (3.5 μm); 0–30% B in 15 min; solvent A, 0.05% TFA in water; solvent B, 0.045% TFA in acetonitrile; 2.0 ml/min; detection at 215 nm. Sample described in Ref. [10].

allow the separation of all compounds of interest. This was true for both the rt-PA and cereal protein samples studied by us. In these and similar cases, it may be necessary to carry out more than one separation of the sample.

References

[1] W.S. Hancock, R.C. Chloupek, J.J. Kirkland and L.R. Snyder, *J. Chromatogr. A*, 686 (1994) 31.
[2] C.T. Mant and R.S. Hodges (Editors), *High-Performance Chromatography of Peptides and Proteins: Separation, Analysis and Conformation*, CRC Press, Boca Raton, FL, 1991.
[3] B.S. Marchylo, D.W. Hatcher and J.E. Kruger, *Cereal Chem.*, 65 (1988) 28.
[4] B.S. Marchylo, D.W. Hatcher and J.E. Kruger, *J. Cereal Sci.*, 9 (1989) 113.
[5] B.S. Marchylo, D.W. Hatcher, J.E. Kruger and J.J. Kirkland, *Cereal Chem.*, 69 (1992) 371.
[6] B.E. Boyes and J.J. Kirkland, *Peptide Res.*, 6 (1993) 249.
[7] V. Ling, A.W. Guzetta, E. Canova-Davis, J.T. Stults, W.S. Hancock, T.R. Covery and B.I. Shushan, *Anal. Chem.*, 63 (1991) 2909.
[8] L.R. Snyder and M.A. Stadalius, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography —Advances and Perspectives*, Vol. 4, Academic Press, New York, 1986, p. 195.
[9] K.D. Nugent, W.G. Burton, T.K. Slattery, B.F. Johnson and L.R. Snyder, *J. Chromatogr.*, 443 (1988) 381.
[10] C.T. Mant and R.S. Hodges, in C.T. Mant and R.S. Hodges (Editors), *High-Performance Chromatography of Peptides and Proteins: Separation, Analysis and Conformation*, CRC Press, Boca Raton, FL, 1991, p. 289.