Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Chromatography A, 1043 (2004) 9–18
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Optimum concentration of trifluoroacetic acid for reversed-phase liquid chromatography of peptides revisited

Y. Chen, A.R. Mehok, C.T. Mant, R.S. Hodges*

Department of Biochemistry and Molecular Genetics, University of Colorado Health Sciences Center, Denver, CO 80262, USA

Available online 18 May 2004

Abstract

Trifluoroacetic acid (TFA) remains the dominant mobile phase additive for reversed-phase high-performance liquid chromatography (RP-HPLC) of peptides after more than two decades since its introduction to this field. Generally, TFA has been employed in a concentration range of 0.05–0.1% (6.5–13 mM) for the majority of peptide separations. In order to revisit the question as to whether such a concentration range is optimum for separations of peptide mixtures containing peptides of varying net positive charge, the present study examined the effect of varying TFA concentration on RP-HPLC at 25 and 70 °C of three groups of synthetic 10-residue synthetic peptides containing either one (+1) or multiple (+3, +5) positively charged groups. The results show that the traditional range of TFA concentrations employed for peptide studies is not optimum for many, perhaps the majority, of peptide applications. For efficient resolution of peptide mixtures, particularly those containing peptides with multiple positive charges, our results show that 0.2–0.25% TFA in the mobile phase will achieve optimum resolution. In addition, the use of high temperature as a complement to such TFA concentration levels is also effective in maximizing peptide resolution.
© 2004 Elsevier B.V. All rights reserved.

Keywords: Mobile phase composition; Trifluoroacetic acid; Peptides

1. Introduction

Since its introduction over two decades ago as an anionic ion-pairing reagent for reversed-phase high-performance liquid chromatography (RP-HPLC), trifluoroacetic acid (TFA) has become the most extensively used mobile phase additive for RP-HPLC of peptides [1–3]. Its efficacy in this role lies in its volatility and UV transparency, coupled with the hydrophobic, negatively charged trifluoroacetate ion (TFA^-) which is able to interact with basic, positively charged amino acid side-chains (Arg, Lys, His), as well as free Nα-amino groups [1–4]. In addition, at low pH values (e.g., pH 2.0), protonation of acidic residues enhances the interaction of peptides with the reversed-phase packing, concomitant with the suppression of free silanol ionization, thereby avoiding undesirable ionic interactions with positively charged peptide solutes [3,5,6].

Favored models for the mechanism of such ion pair separations involve either formation of ion-pairs with the sample solute in solution followed by retention of the solute molecules on a reversed-phase packing [7,8] or a dynamic ion-exchange event whereby the ion-pairing reagent is first retained by the reversed-phase column and then solute molecules exchange ions with the counterion associated with the sorbed ion-pair reagent [8–11]. Whatever the mechanism, the resolving power of negatively charged, anionic ion-pairing reagents such as TFA is effected through their interaction with the aforementioned positively charged groups in a peptide [3,4]. Indeed, hydrophobic anions such as TFA^- will not only neutralize the positively charged groups, thereby decreasing peptide hydrophilicity, but will increase further the affinity of the peptides for the reversed-phase sorbent [4].

In the past, concentrations of TFA in mobile phases (aq. TFA/acetonitrile mobile phase systems being the most commonly employed for peptides [1–4]) have mainly been limited to a range of 0.05–0.1% (v/v). However, an early study in our laboratory [4] demonstrated the potential use of varying TFA concentrations (0.01–0.8%) on peptide selectivity, where increasing TFA concentration resulted in an increase in peptide retention time; further, the greater the number of positive charges on a peptide, the greater the increase in peptide retention, demonstrating potential manipulation of peptide elution profiles by varying anionic

* Corresponding author. Tel.: +1-303-315-8837; fax: +1-303-315-1153.
E-mail address: robert.hodges@uchsc.edu (R.S. Hodges).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.03.070

counterion concentration. Such an approach has also been reported for the preparative RP-HPLC separation of very hydrophilic, histidine-rich peptides, where 1% TFA was employed to ensure retention of the peptides by the RP-HPLC packing [12]. Considering the range of positively charged peptides which may be present in peptide mixtures—in proteomic applications, for instance, where protein digests may contain thousands of peptides with multiple charges—we believe the question of whether traditionally employed concentrations of TFA in mobile phase systems are indeed optimized for general peptide applications should be revisited.

Routine use of higher TFA concentrations has generally been avoided in the past, perhaps partly due to concerns of stationary phase degradation, e.g., cleavage of alkyl chains from silica-based packings *via* acid hydrolysis of the siloxane bond linking the stationary phase functional group with surface silanols [13,14]. However, with the advent of reversed-phase packings with excellent stability towards both acidic mobile phases and high temperature [15–17], such concerns have been overcome. Thus, the present study determines the effect of varying TFA concentration and temperature on RP-HPLC of three groups of synthetic model peptides, these groups containing peptides of +1, +3 or +5 net charge. From the retention behavior of these peptides, conclusions could be drawn about optimum mobile phase conditions for sample mixtures containing peptides of varying net charge.

2. Experimental

2.1. Materials

TFA was obtained from Halocarbon Products (River Edge, NJ, USA). HPLC-grade acetonitrile was purchased from EM Science (Gibbstown, NJ, USA). HPLC-grade water was obtained from EMD Chemicals (Gibbstown, NJ, USA).

2.2. Instrumentation

Analytical RP-HPLC runs were carried out on an HP 1100 Liquid Chromatograph (Hewlett-Packard, Avondale, PA, USA), coupled with an HP 1100 series diode array detector and thermostatted column compartment, HP Vectra XA computer and HP LaserJet 5 printer. Peptide synthesis was carried out on an Applied Biosystems Peptide synthesizer Model 430A (Foster City, CA, USA).

2.3. Column and HPLC conditions

Analytical RP-HPLC runs were carried out on a Zorbax SB300-C_8 column (150 mm × 2.1 mm i.d.; 5 μm particle size, 300 Å pore size) from Agilent Technologies (Little Falls, DE, USA), using a linear AB gradient (1% acetonitrile/min) at a flow-rate of 0.25 ml/min, where eluent A was 2–32 mM aq. TFA and eluent B was the corresponding concentration of TFA in acetonitrile; runs were carried out at 25 and 70 °C. Approximately 1 μmol of each of the peptides in the 10-peptide mixtures were injected in a total sample volume of 10 μl.

2.4. Peptide synthesis and purification

Synthesis of the peptides was carried out by standard solid-phase synthesis methodology using N^{α}-*tert*-butyloxycarbonyl (*t*-Boc) chemistry on MBHA (methylbenzhydrylamine) resin (0.97 mmol/g) as described previously [18]. The crude peptides were purified by preparative RP-HPLC on an Applied Biosystems 400 solvent-delivery system connected to a 783A programmable absorbance detector. Amino acid analyses of purified peptides were carried out on a Beckman Model 6300 amino acid analyzer (Beckman Instruments, Fullerton, CA, USA) and the correct primary ion molecular masses of peptides were confirmed by mass spectrometry on a Fisons VG Quattro electrospray mass spectrometer (Fisons, Point-Claire, Canada). Note that 0.2% TFA can be used in LC–MS–MS with detection at the femtomole level.

2.5. Calculation of resolution (R_s)

Resolution was calculated through the equation:

$$R_{\mathrm{s}} = \frac{1.176\Delta t_{\mathrm{R}}}{W_1 + W_2}$$

where Δt_{R} is the difference in retention time between two peptide peaks (1 and 2) and W_1 and W_2 are their peak widths at half height. This equation is satisfied if the units of retention time and peak width are the same, such as minutes. The peak widths at half height (in time units) used in this study were determined using the HP ChemStation for LC Systems software package Rev.07.01.

3. Results

3.1. Design of synthetic model peptides

We have always believed that studies attempting to equate peptide elution behavior in HPLC generally, and RP-HPLC specifically, with varying run parameters is best achieved by initial studies using defined model peptide systems, the results of which can then be extrapolated to peptides as a whole. Thus, we have designed and synthesized three groups of model peptides exhibiting variations in hydrophobicity and net positive charge (Table 1). From Table 1, each group of peptides contains 10 peptides with the same net positive charge, arising from the presence of a single lysine residue (+1 group), two lysine residues and an arginine residue (+3 group) or two lysine residues, two arginine residues and a free N-terminal α-amino group (+5 group).

Table 1
Sequences and names of the peptides in this study

Peptide group[a]	Peptide name	Peptide sequence[b]	Increase in the number of carbon atoms[c]
+1	1a	Ac--GG**GG**GLGLGK--amide	0
	1b	Ac--GG**AG**GLGLGK--amide	1
	1c	Ac--GG**AA**GLGLGK--amide	2
	1d	Ac--GG**VG**GLGLGK--amide	3
	1e	Ac--GG**VA**GLGLGK--amide	4
	1f	Ac--GG**IG**GLGLGK--amide	4
	1g	Ac--GG**IA**GLGLGK--amide	5
	1h	Ac--GG**VV**GLGLGK--amide	6
	1i	Ac--GG**IV**GLGLGK--amide	7
	1j	Ac--GG**II**GLGLGK--amide	8
+3	3a	Ac--GR**GG**KLGLGK--amide	0
	3b	Ac--GR**AG**KLGLGK--amide	1
	3c	Ac--GR**AA**KLGLGK--amide	2
	3d	Ac--GR**VG**KLGLGK--amide	3
	3e	Ac--GR**VA**KLGLGK--amide	4
	3f	Ac--GR**IG**KLGLGK--amide	4
	3g	Ac--GR**IA**KLGLGK--amide	5
	3h	Ac--GR**VV**KLGLGK--amide	6
	3i	Ac--GR**IV**KLGLGK--amide	7
	3j	Ac--GR**II**KLGLGK--amide	8
+5	5a	NH_3^+--RR**GG**KLGLGK--amide	0
	5b	NH_3^+--RR**AG**KLGLGK--amide	1
	5c	NH_3^+--RR**AA**KLGLGK--amide	2
	5d	NH_3^+--RR**VG**KLGLGK--amide	3
	5e	NH_3^+--RR**VA**KLGLGK--amide	4
	5f	NH_3^+--RR**IG**KLGLGK--amide	4
	5g	NH_3^+--RR**IA**KLGLGK--amide	5
	5h	NH_3^+--RR**VV**KLGLGK--amide	6
	5i	NH_3^+--RR**IV**KLGLGK--amide	7
	5j	NH_3^+--RR**II**KLGLGK--amide	8

[a] The charge of the peptide is shown at pH 2.0.
[b] The different amino acid substitutions are shown in bold letters.
[c] The increase of the number of carbon atoms is assigned taking peptides 1a, 3a and 5a as zero.

Within each peptide group, hydrophobicity varies only subtly between adjacent peptides, i.e., peptide hydrophobicity varying by just one methyl or methylene group (equivalent to an increase of one carbon atom) from one peptide to the next. The presence of several glycine residues ensured negligible secondary structure for these peptides [19,20] (i.e., they have a "random coil" configuration), to avoid complications in interpretation of data due to selectivity differences in peptide RP-HPLC retention behavior arising from conformational variations [21,22]. The 10-residue length of the peptides was chosen to mimic the size of an average peptide fragment arising from proteolytic digests of proteins. Peptides are denoted by charge and relative hydrophobicity order, e.g., the peptide with one positive charge and the lowest hydrophobicity within this +1 group (a –GG– substitution; Table 1) is denoted 1a; the peptide with three positive charges and the highest hydrophobicity within this +3 group (a –II– substitution; Table 1) is denoted 3j, etc.

3.2. RP-HPLC stationary phase

The Zorbax SB-300C$_8$ ("SB" denoting "Stable Bond") is prepared from monofunctional *n*-octylsilane based on protecting the siloxane bond between the silica and the C$_8$ group with bulky side groups, in this case two isopropyl groups [15–17]. This packing was originally designed to protect the siloxane bond from acid hydrolysis at low pH [15–17] and has shown excellent thermal stability at pH 2 [22–27].

3.3. Effect of TFA concentration on elution behavior of model peptide mixtures

The effect of TFA concentration on the RP-HPLC retention behavior of mixtures of positively charged peptides was determined by running the three 10-peptide groups (+1, +3 and +5; Table 1) in aq. TFA/acetonitrile mobile phases containing 2, 4, 8, 16 and 32 mM TFA (equivalent to a range of ∼0.016–0.25% TFA, i.e., encompassing the 0.05–0.1% TFA range traditionally used for such separations) at 25 and 70 °C. It should be noted that we chose to express TFA concentrations in mM *versus* percentage in order to be able to make a direct comparison of the effectiveness of TFA with alternative ion-pairing reagents, the subject of a separate study. The pH value of the aq. TFA (eluent A) ranged from pH 2.8 (2 mM TFA) to pH 1.7 (32 mM), which we refer to generally as pH 2. Note that even the highest pH value (pH 2.8 for 2 mM aq. TFA) is far enough below the pK_a values of the positively charged groups in the peptides so as not to affect the full positive charge on the peptides; in addition, if any underivatized silanol groups (pK_a ∼4.0) remained on the Stable Bond packing, they also would remain protonated (i.e., neutral) under the RP-HPLC conditions used in the present study, thus preventing any potential undesirable electrostatic interactions between the positively charged peptides and the hydrophobic stationary phase. Elution time data for all peptides at 25 and 70 °C are presented in Table 2.

Fig. 1 compares the results obtained at 70 °C when running the three groups of peptides in the 2, 8 and 32 mM TFA mobile phase systems. From Fig. 1, increasing TFA concentration generally results in increasing peptide retention time and improved peak shape. In addition, this effect of increasing TFA concentration is more marked the greater the positive charge on the peptides, i.e., +1 group < +3 group < +5 group. This effect is especially dramatic for the +5 group, where early eluted peptides, in particular, showed severe tailing and poor peak shape at low (2 mM) TFA concentration.

An interesting observation from Fig. 1 is the effect of TFA concentration on the elution time range of the peptides, i.e., Δt_R (j analogue − a analogue) for each peptide group. At 32 mM TFA, the values were 11.8 min (+1 group), 9.8 min (+3 group) and 7.2 min (+5 group), i.e., the larger the number of positive charges, the smaller the elution range. These Δt_R values for the +1 and +3 groups remained essentially

Table 2
Retention times of +1, +3 and +5 peptides in different TFA concentrations

Peptide group	TFA (mM)	Temperature (°C)	Retention time (min)									
			a[a]	b	c	d	e	f	g	h	i	j
+1	2	25	17.89	19.04	20.09	22.00	23.16	24.16	25.43	24.87	27.04	28.96
		70	16.40	17.63	18.78	20.77	22.03	22.93	24.11	24.11	26.32	28.29
	4	25	18.20	19.34	20.37	22.28	23.44	24.44	25.71	25.17	27.34	29.25
		70	16.71	17.93	19.06	21.06	22.33	23.23	24.44	24.44	26.62	28.59
	8	25	18.48	19.59	20.60	22.50	23.65	24.66	25.92	25.37	27.53	29.44
		70	16.95	18.15	19.27	21.27	22.53	23.44	24.66	24.66	26.84	28.81
	16	25	18.78	19.88	20.87	22.77	23.93	24.94	26.20	25.68	27.83	29.75
		70	17.28	18.47	19.57	21.56	22.83	23.74	24.99	24.99	27.15	29.11
	32	25	19.08	20.16	21.14	23.03	24.19	25.21	26.47	25.98	28.13	30.06
		70	17.56	18.73	19.82	21.81	23.08	23.99	25.26	25.26	27.42	29.40
+3	2	25	15.77	16.67	17.71	19.17	19.88	21.03	21.62	21.62	23.42	25.11
		70	13.96	14.87	15.99	17.51	18.34	19.35	20.11	20.48	22.13	23.82
	4	25	17.02	17.85	18.82	20.22	20.94	22.07	22.69	22.69	24.45	26.13
		70	15.12	16.01	17.09	18.57	19.41	20.42	21.20	21.53	23.18	24.89
	8	25	17.92	18.72	19.65	21.00	21.72	22.84	23.49	23.49	25.22	26.90
		70	15.94	16.82	17.87	19.33	20.19	21.19	22.00	22.30	23.97	25.69
	16	25	19.06	19.84	20.74	22.05	22.80	23.89	24.60	24.60	26.32	28.01
		70	17.12	17.99	19.02	20.45	21.32	22.30	23.16	23.42	25.13	26.85
	32	25	19.97	20.74	21.62	22.89	23.68	24.75	25.47	25.58	27.24	28.95
		70	18.01	18.87	19.90	21.30	22.21	23.16	24.09	24.33	26.07	27.81
+5	2	25	10.33	11.40	12.82	14.61	14.98	16.34	16.34	17.38	18.35	19.96
		70	9.35	9.35	9.35	11.73	12.42	13.71	14.14	15.15	16.20	17.84
	4	25	13.94	14.46	15.33	16.70	17.03	18.23	18.23	19.14	20.05	21.57
		70	10.25	11.32	12.69	14.17	14.73	15.84	16.19	17.08	18.07	19.61
	8	25	16.35	16.75	17.40	18.59	18.90	20.02	20.02	20.84	21.71	23.18
		70	13.25	13.90	14.90	16.10	16.63	17.63	17.97	18.79	19.73	21.25
	16	25	18.70	19.03	19.56	20.65	20.94	22.02	22.02	22.75	23.61	25.06
		70	15.91	16.41	17.21	18.27	18.75	19.71	20.02	20.80	21.73	23.22
	32	25	20.21	20.53	20.99	22.08	22.34	23.43	23.43	24.13	25.01	26.48
		70	17.57	18.02	18.76	19.80	20.27	21.22	21.52	22.27	23.22	24.73

[a] For peptide denotions, see Table 1; RP-HPLC conditions, see Section 2.3.

constant over the entire range of TFA concentrations examined (2–32 mM); for the +5 groups, these values remained essentially constant over the 8–32 mM TFA range. Also from Fig. 1, an increase in TFA concentration dramatically improved resolution for specific peptide pairs. Thus, for the +5 group, peptides 5f and 5g are almost completely separated to baseline at 32 mM TFA but poorly resolved at lower TFA concentrations. Similarly, for the +3 group, peptides 3g and 3h are almost completely separated to baseline at 32 mM TFA. Further, for the +1 group, 1g and 1h are coeluted for the entire TFA concentration range even though there has been an increase in overall resolution of the peptide mixture at 32 mM TFA. There are also subtle selectivity differences between peptides with the same hydrophobicity difference within the three groups of peptides, e.g., compare the g and h analogues, which are not resolved in the +1 group (1g/1h) but are resolved in the +3 (3g/3h) and +5 (5g/5h) groups.

Finally, a critical conclusion from the results presented in Fig. 1 is that, for the efficient resolution of all three groups of peptides (at 70 °C), a TFA concentration of 32 mM (∼0.25%) is required. Similar results were also obtained at 25 °C (data not shown).

3.4. Effect of temperature on elution behavior of model peptide mixtures

Fig. 2 illustrates the effect of temperature on elution behavior of the three groups of peptides in the presence of 32 mM TFA. This concentration of TFA was maintained due to its producing the best overall resolution for all three groups of peptides in Fig. 1. From Fig. 2, an increase in temperature reduces peptide retention time, due to an enhancement of the mass-transfer rate of the peptide solutes between the stationary and mobile phases [17,28–30]. Advantages of

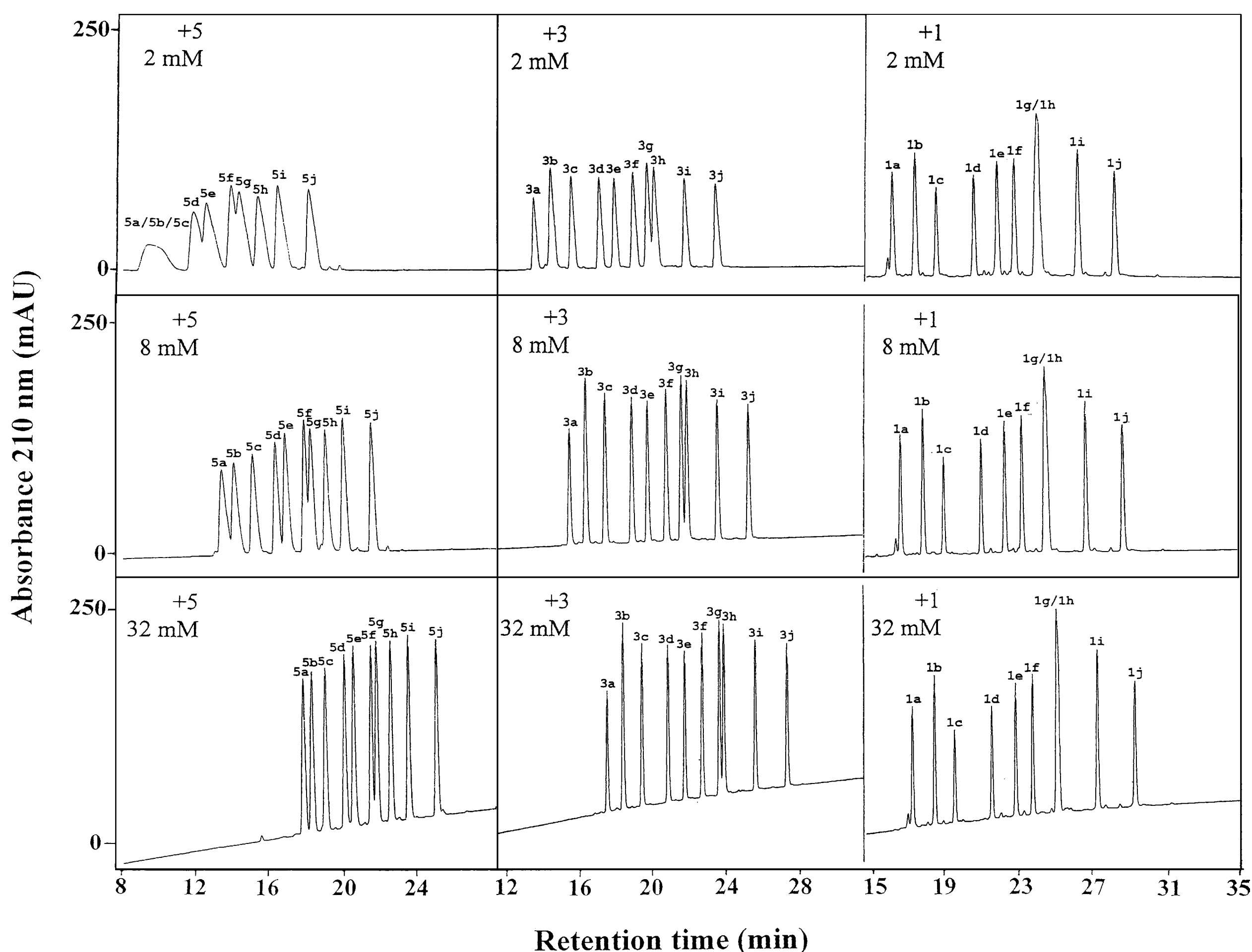

Fig. 1. Effect of TFA concentration on RP-HPLC retention behavior of positively charged model peptide mixtures. Conditions: linear AB gradient (1% acetonitrile/min) at a flow-rate of 0.25 ml/min, where eluent A is 2, 8 or 32 mM aq. TFA and eluent B is the corresponding TFA concentration in acetonitrile; temperature, 70 °C. The structures and denotions of the peptides are shown in Table 1; +5, +3 and +1 refer to the net charge of all peptides in the peptide mixtures.

varying temperature may be seen in selectivity changes at 25 and 70 °C. Thus, peptide pairs 5f/5g (+5 group) and 3g/3h (+3 group) are coeluted at 25 °C but are resolved at 70 °C; conversely, peptides 1h and 1g (+1 group) are resolved to baseline at 25 °C but are completely coeluted at 70 °C. In addition, the peptide pair 5a/5b and 5d/5e (+5 group) is better resolved at 70 °C compared to 25 °C.

Interestingly, the elution range of the peptides, i.e., Δt_R (j analogue − a analogue) appears to be affected by a temperature change. Thus, at 25 °C, Δt_R values are 6.3, 9.0 and 11.0 min for the +5, +3 and +1 groups, respectively; in contrast, these values have increased to 7.2, 9.9 and 11.8 min, respectively, at 70 °C, i.e., an average increase for all these peptide groups of ~0.9 min.

Finally, it should be noted that the option to be able to use a relatively high TFA concentration (32 mM) combined with high temperatures for manipulation of elution profiles of peptide mixtures highlights well the advantages of chemically and thermally resistant silica-based RP-HPLC packings.

3.5. Effect of nearest-neighbor effects on elution behavior of model peptide mixtures

Deviations from expected elution behavior for small peptides are generally explained in terms of sequence-specific effects, which can be divided into two categories—nearest-neighbor and conformation effects [31]. The former implies that such effects are amino acid sequence-dependent, but independent of differences in secondary structure; in comparison, amino acid sequence-dependent conformational effects would be an apparent reduction or enhancement of the overall hydrophobicity of the peptide as a result of the peptide adopting an unique conformation on interacting with the stationary phase, compared to the hydrophobicity of the peptide if it existed as a random coil, i.e., lacking an unique conformation [31]. The random coil nature of the model peptides in the present study, assured by the presence of multiple glycine residues, suggests that subtle selectivity differences during RP-HPLC are likely due to nearest-neighbor effects within specific peptides and/or the environment

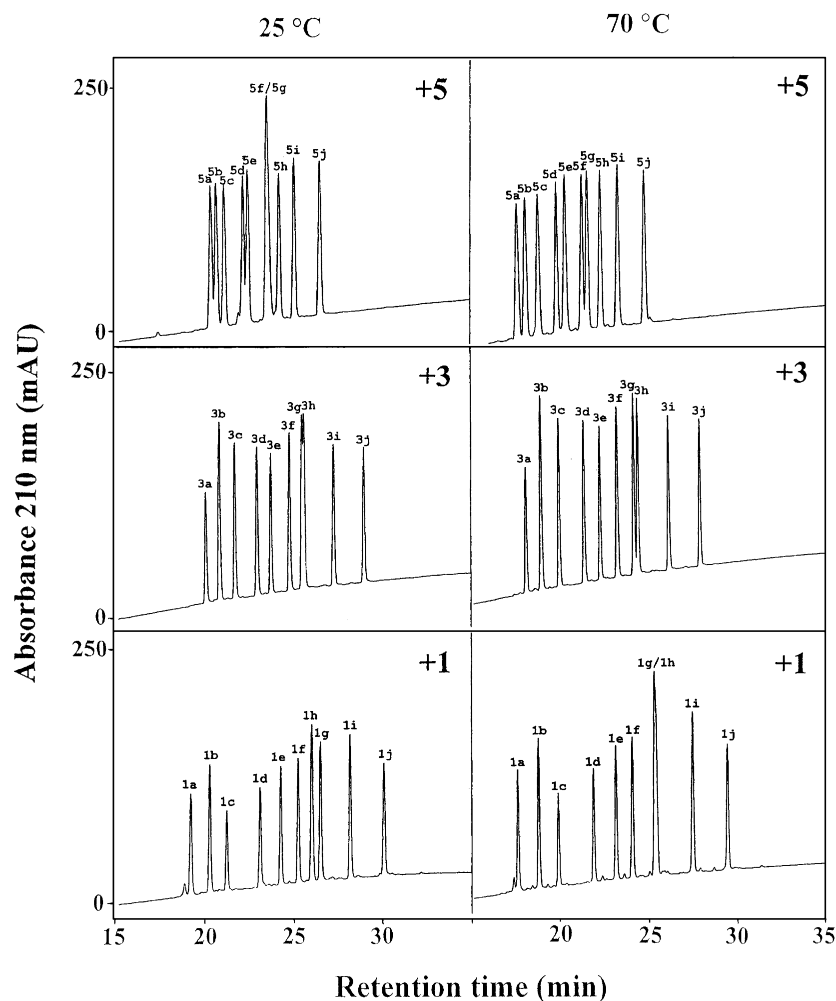

Fig. 2. Effect of temperature on RP-HPLC retention behavior of positively charged model peptide mixtures. Conditions: linear AB gradient (1% acetonitrile/min) at a flow-rate of 0.25 ml/min, where eluent A is 32 mM aq. TFA and eluent B is 32 mM TFA in acetonitrile. The structures and denotions of the peptides are shown in Table 1; +5, +3 and +1 refer to the net charge of all peptides in the peptide mixtures.

within which residue substitutions are made. Thus, even through the peptide analogues in all three groups of peptides have the same series of residue substitutions (Table 1), the environment surrounding these substitutions varies substantially. For instance, for the +5 group, the substitutions are made within the sequence $^{+}H_3N$–RR–X–X–K– (where X–X denotes substituted residues), i.e., within a highly charged environment, in addition to three adjacent positive charges at its N-terminus; for the +3 group, substitutions are made within the sequence Ac–GR–X–X–K–, i.e., within a much lesser charged environment than the +5 group; finally, for the +1 group, substitutions are made within the sequence Ac–GG–X–X–G–, i.e., with no ionizable groups close to the substitution positions.

From Fig. 1, peptide analogues g and h, which differ by a full carbon atom (partial sequence of –GIAG– for g and –GVVG– for h; Table 1), were not resolved in the +1 group (1g/1h) at any TFA concentration (2–32 mM TFA) at 70 °C; however, they were baseline resolved at 25 °C (Fig. 2). Interestingly, the same sequence variations for the +3 group (–RIAK– for 3g and –RVVG– for 3h) could not be resolved at 25 °C but could be resolved at 70 °C (Fig. 2). For the +5 group (–RIAK– for 5g and –RVVG– for 5h), the two peptides are readily resolved at 25 and 70 °C (Fig. 2), where the only difference between the +3 and +5 groups is the replacement of the N-terminal glycine residue in the +3 groups with an arginine residue with a free α-amino group in the +5 group. Such a result illustrates the sensitivity of RP-HPLC to subtle changes in sequence not immediately adjacent to the dipeptide sequence where the hydrophobicity changes. Further, what is even more interesting is the separation of peptides 1g and 1h at 25 °C (Fig. 2). Peptide 1h has a greater intrinsic hydrophobicity than 1g by 1 carbon atom out of the 10-residue sequence (Table 1), yet peptide 1h is eluted prior to 1g at 25 °C while the two peptides are coeluted at 70 °C (Fig. 2). This result suggests that nearest-neighbor effects are greater at 25 °C and such effects are being diminished at high temperature.

Another interesting example of a nearest-neighbor effect lies in the behavior of the e and f analogues of the three pep-

tide groups. Thus, e analogues contain the sequence –VA–, whilst f analogues contain the sequence –IG–, i.e., they were designed to contain the same number of carbon atoms (four) in the substitution positions and, thus, the same apparent intrinsic hydrophobicity, where there is an increase of one methyl group in going from valine to isoleucine and a decrease of one methyl group in going from alanine to glycine (Table 1). Regardless of the surrounding residues (–GVAG– and –GIGG– in the +1 group; –RVAK– and –RIGK– in the +3 group and +5 group; Table 1), the peptides are readily separated at both 25 and 70 °C (Fig. 2). It is perhaps significant that the e analogues (with the –VA– substitution) are consistently eluted prior to the f analogues (with the –IG– substitution). Thus, the inherent hydrophobicity of the isoleucine side-chain (f analogues), adjacent as it is to glycine with no appreciable side-chain, is likely to be fully expressed on interaction with the hydrophobic stationary phase. In contrast, instead of being additive, it is likely that the combined hydrophobicity of the valine and alanine side-chains (e analogues) is not being fully expressed, i.e., the apparent hydrophobicity of this dipeptide is being diminished relative to –IG– of the f analogues, perhaps due to "shielding" of the hydrophobicity of one residue by the other via conformational constraints between these adjacent residues.

3.6. Effect of TFA concentration and temperature on elution characteristics of model peptides

Fig. 3 summarizes graphically the effect of increasing TFA concentration (expressed as $\log_{10}$[TFA]) on Δt_R (32–8 mM) (i.e., the difference in peptide retention time between that obtained at a concentration of 32 mM TFA compared to 8 mM TFA), peptide peak width at half height ($W_{1/2}$) and peptide resolution. To simplify interpretation of data, only profiles obtained from selected peptide data are shown: thus, only values obtained for 5h/5i and 5i/5j (+5 group); 3b/3c, 3d/3e and 3i/3j (+3 group); and 1b/1c, 1d/1e and 1i/1j (+1 group) are presented. However, the data presented in Fig. 3 represents the observed elution behavior for all peptide analogues and can be viewed as summarizing general rules for effect of TFA concentration on peptides of varying net charge.

From Fig. 3, Δt_R (32–8 mM values) are essentially independent of TFA concentration for all three peptide groups at both 25 and 70 °C. Table 3 shows the change in peptide retention times between the 8 and 32 mM TFA systems at both 25 and 70 °C. Due to the comparatively poor retention of the +5 group peptides at concentrations of 2 mM TFA (Fig. 1) and 4 mM TFA (data not shown), 8 mM was chosen as the lower TFA concentration limit to ensure accuracy of data. Note that the average Δt_R/positive charge values are essentially constant at both 25 and 70 °C for all three groups, albeit there is an increase in the values as the net charge increases, i.e., $+1 < +3 < +5$. This increase reflects the earlier observation (Fig. 1) that the elution range of the peptides (t_R: j analogue − a analogue) increases with increasing net positive charge.

Clearly, peptide peak width is decreasing with increasing TFA concentration (as well as increasing temperature), this effect being more marked the higher the net positive charge on the peptides. Indeed, the effect on peak width by an increase in both TFA concentration and temperature is most dramatic for the +5 group, as had been noted from the elution profiles shown in Fig. 1. The overall general trend of decreasing peak width with an increase in TFA concentration and temperature is also illustrated in Table 4 which reports the difference in $W_{1/2}$ values obtained between 8 and 32 mM TFA (i.e., $W_{1/2}$ at 32 mM − $W_{1/2}$ at 8 mM). The average $W_{1/2}$ value/net positive charge is presented simply to highlight the effect of temperature. Thus, over this entire TFA concentration range, the average $W_{1/2}$/charge is always lower (i.e., this value is more negative) at 70 °C compared to 25 °C.

Finally, from Fig. 3, for all three peptide groups, increasing TFA concentration clearly increases resolution of adjacent peptide pairs. In addition, as reflected by the steeper slopes of the 70 °C data, increasing the temperature from 25 °C also increases peptide resolution over the entire TFA concentration range. The effects of these two run parameters is underlined in Table 5, which reports the relative increase in peptide resolution at 70 °C *versus* 25 °C on increasing the TFA concentration from 2 to 32 mM TFA. Thus, for instance, for the +1 group, resolution increases 1.21-fold at 32 mM TFA compared to 2 mM TFA at 25 °C for the 1b/1c peptide pair compared to 1.44-fold at 70 °C; these values are 1.32-fold (25 °C) and 1.57-fold (70 °C) for the 1d/1e peptide pair; and 1.42-fold (25 °C) and 1.60-fold (70 °C) for the 1i/1j peptide pair. The same trend is also observed for the +3 and +5 group peptides. Also from Table 5, it is clear that, the higher the net positive charge on the peptides, the greater the improvement in resolution on raising the TFA concentration from 2 to 32 mM TFA. Thus, taking the i/j analogues as an example, the resolution of 1i/1j, 3i/3j and 5i/5j peptide pairs improves by 1.42-, 1.83- and 2.07-fold, respectively, at 25 °C; at 70 °C, the resolution of these peptide pairs improves 1.60-, 2.18- and 2.54-fold, respectively. Finally, Table 5 again highlights interesting selectivity differences with increasing TFA concentration. Such selectivity differences vary from peptide pair to peptide pair and, thus, the improvement in resolution between peptide pairs is not identical in all cases. A clear example of this from Table 5 is that the relative increase in resolution at 70 °C for 3g/3h is significantly lower (1.44) than the general range of the peptides (2.00–2.45), whereas the 3f/3g pair is somewhat higher (2.69), i.e., the elution behavior is causing these anomalies. Thus, for the 3g/3h peptide pair, the difference in retention times between the peptides has actually decreased on raising the TFA concentration from 2 to 32 mM Table 2), although the concomitant decreases in peak widths (Table 4) still lead to an overall relative increase in resolution (1.44), albeit significantly less than might be expected. In contrast,

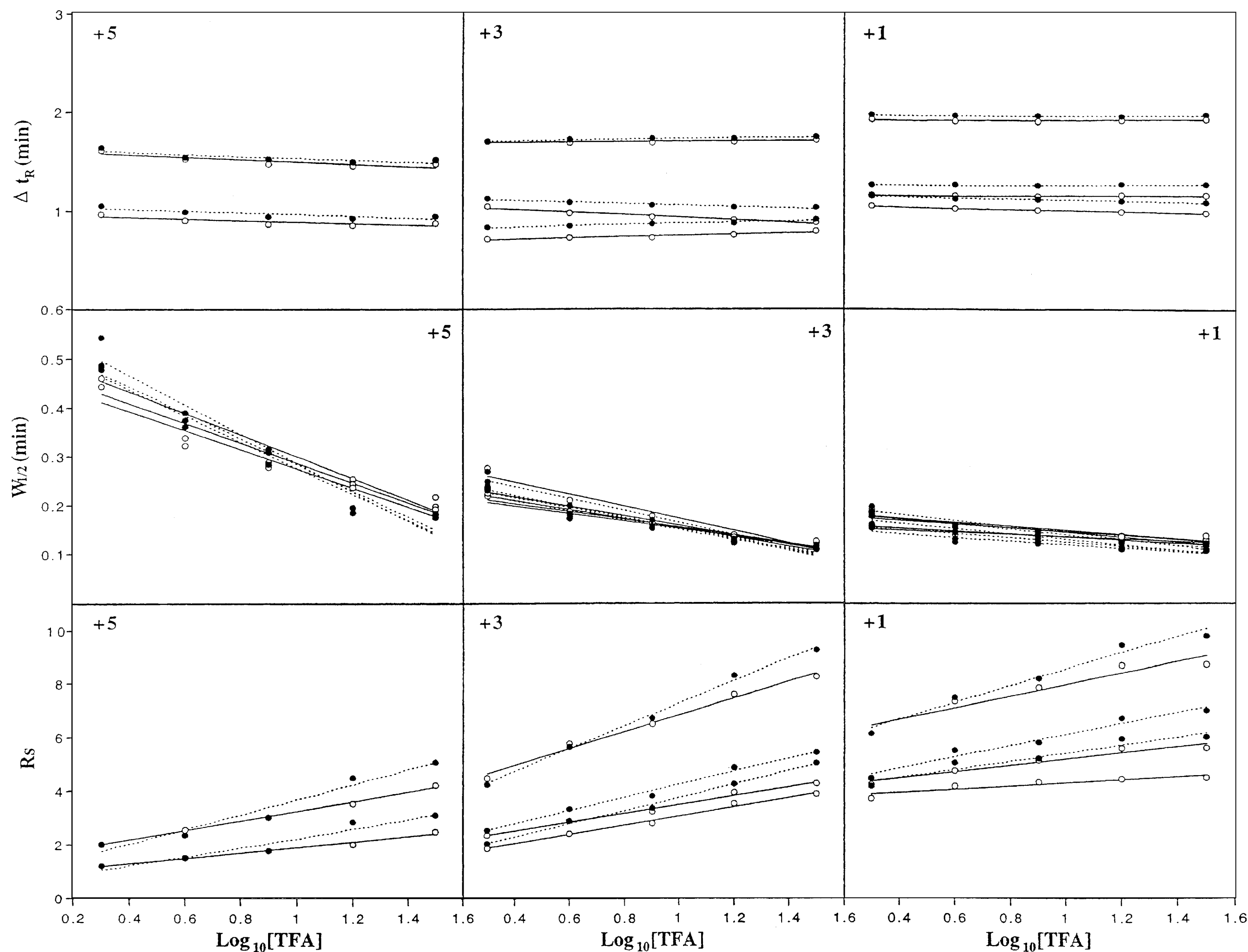

Fig. 3. Effect of TFA concentration on retention characteristics of model positively charged peptides at 25 and 70 °C. Δt_R denotes difference in retention time between adjacent peptides; $W_{1/2}$ denotes peak width at half height of peptides; R_s denotes resolution of adjacent peptides. Open symbols with solid lines represent results obtained at 25 °C; closed symbols with dotted lines represent results obtained at 70 °C. The +5 group peptides are represented by peptide pairs 5h/5i and 5i/5j; the +3 group peptides are represented by peptide pairs 3b/3c, 3d/3e and 3i/3j; the +1 group peptides are represented by peptide pairs 1b/1c, 1d/1e and 1i/1j. Sequences of all peptides are shown in Table 1.

Table 3
Difference in retention times of +1, +3 and +5 peptides between 8 and 32 mM TFA

Peptide group	Temperature (°C)	$\Delta t_{R(32-8\,mM)}$ (min)[a]										Average[b]	Average/charge[c]
		a[d]	b	c	d	e	f	g	h	i	j		
+1	25	0.60	0.57	0.54	0.53	0.54	0.55	0.55	0.61	0.60	0.62	0.57	0.57
	70	0.61	0.58	0.55	0.54	0.55	0.55	0.60	0.60	0.58	0.59	0.58	0.58
+3	25	2.05	2.02	1.97	1.89	1.96	1.91	1.98	2.09	2.02	2.05	1.99	0.66
	70	2.07	2.05	2.03	1.97	2.02	1.97	2.09	2.03	2.10	2.12	2.05	0.68
+5	25	3.86	3.78	3.59	3.49	3.44	3.41	3.41	3.29	3.30	3.30	3.49	0.70
	70	4.32	4.12	3.86	3.70	3.64	3.59	3.55	3.48	3.49	3.48	3.72	0.74

[a] Denotes the difference of peptide retention times between 8 and 32 mM TFA.
[b] Average denotes the average values of the $\Delta t_{R(32-8\,mM)}$ of 10 peptides analogues in the same group.
[c] Average/charge denotes the average values of the $\Delta t_{R(32-8\,mM)}$ of 10 peptide analogues in the same group divided by the number of charges on the peptides.
[d] For peptide denotions, see Table 1; RP-HPLC conditions, see Section 2.3.

Table 4
Difference of peak width at half height of +1, +3 and +5 peptides between 8 and 32 mM TFA

Peptide group	Temperature (°C)	$\Delta W_{1/2(32-8\,mM)}$ (min)[a]										Average[b]	Average/charge[c]
		a[f]	b	c	d	e	f	g	h	i	j		
+1	25	−0.0185	−0.0119	−0.0057	−0.0074	−0.0112	−0.0141	−0.0168	−0.0171	−0.0202	−0.0055	−0.0128	−0.0128
	70	−0.0217	−0.0252	−0.0144	−0.0172	−0.0258	−0.0293	–[d]	–	−0.0272	−0.0187	−0.0224	−0.0224
+3	25	−0.0451	−0.0513	−0.0440	−0.0344	−0.0307	−0.0366	–	–	−0.0323	−0.0294	−0.0380	−0.0127
	70	−0.0415	−0.0523	−0.0474	−0.0466	−0.0402	−0.0476	−0.0446	−0.0424	−0.0425	−0.0380	−0.0443	−0.0148
+5	25	–	–	–	–	–	–	–	−0.0852	−0.0710	−0.0862	−0.0808	−0.0162
	70	–[e]	−0.1865	−0.1732	−0.1774	−0.0928	–	–	−0.1319	−0.1325	−0.1078	−0.1432	−0.0286

[a] Denotes the difference of peptide peak width at half height between 8 and 32 mM TFA.
[b] Average denotes the average values of the $\Delta W_{1/2(32-8\,mM)}$ of the corresponding peptide analogues in the same group.
[c] Average/charge denotes the average values of the $\Delta W_{1/2(32-8\,mM)}$ of the corresponding peptide analogues in the same group divided by the number of charges on the peptides.
[d] Dashes denote the coeluted peaks or the poorly resolved peaks, for which the peak width at half height cannot be determined.
[e] Peptide 5a is not retentive enough in 8 mM TFA at 70 °C and, therefore, is not included in this table.
[f] For peptide denotions, see Table 1; RP-HPLC conditions, see Section 2.3.

Table 5
Relative increase in peptide resolution (R_s) between 2 and 32 mM TFA[a]

Peptide group	Temperature (°C)	Relative increase in R_s values[b]								
		a–b[c]	b–c	c–d	d–e	e–f	f–g	g–h	h–i	i–j
+1	25	1.31	1.21	1.24	1.32	1.13	1.54	1.27	1.51	1.42
	70	1.47	1.44	1.46	1.57	1.63	–[d]	–	–	1.60
+3	25	1.84	1.81	1.74	2.07	1.77	–	–	–	1.83
	70	2.06	2.12	2.13	2.45	2.00	2.69	1.44	2.26	2.18
+5	25	–	–	–	–	–	–	–	2.06	2.07
	70	–	–	–	–	–	–	–	2.58	2.54

[a] RP-HPLC conditions, linear AB gradient (1% acetonitrile/min) at a flow-rate of 0.25 ml/min, where eluent A is 2 or 32 mM aq. TFA and eluent B is the corresponding TFA concentration in acetonitrile.
[b] Calculated by the expression (R_s of peptide pair at 2 mM TFA)/(R_s of peptide pair at 32 mM TFA).
[c] Denotes adjacent peptide pair from which R_s values were derived; sequences shown in Table 1.
[d] Denotes situations where poorly resolved or coeluted peaks prevented measurement of R_s.

the difference in retention time between 3f and 3g has increased with increasing TFA concentration; thus, with the concomitant decrease in peak widths, the relative increase in resolution (2.69) is somewhat greater than the remaining adjacent peptide pairs. Such results again confirm the advantage of being able to employ high TFA concentration and/or high temperature to improve resolution of peptide mixtures.

Raising the TFA concentration further to 64 and 128 mM was found to be impractical due to considerable loss of peak detection sensitivity at high TFA concentrations. Thus, at 64 mM TFA, peptide peak areas were already somewhat smaller than those observed at 32 mM TFA; at 128 mM TFA >80% of peak area compared to 32 mM TFA had been lost. Such results likely arise from the strong UV-absorbing characteristics of TFA at high concentration interfering with peptide bond detection at 210 nm. However, considering the excellent results achieved with 32 mM TFA concomitant with only incremental further improvements in peptide resolution at higher TFA concentrations, this loss of detection sensitivity at TFA concentrations >32 mM TFA is of no practical concern.

From the results of this study, it is apparent that the concentrations of TFA generally employed for RP-HPLC of peptides (0.05–0.1%, i.e., ∼6.5–13 mM) are somewhat at the low end of a favorable concentration range for such purposes, particularly for peptides containing multiple charges (e.g., +5 group in Fig. 1). Indeed, we suggest that the concentration of TFA employed for general peptide applications should be altered to the 0.2–0.25% range (∼26–32 mM) to ensure optimum peptide resolution, particularly considering the now ready availability of stable, silica-based RP-HPLC packings.

4. Conclusions

The present study has investigated the effect of varying TFA concentration and temperature on RP-HPLC of three groups of synthetic model peptides containing either one (+1) or multiple (+3, +5) positively charged groups. The results clearly show that the traditional range of TFA concentrations employed for peptide studies (0.05–0.1%) is not

optimum for many, perhaps most, peptide applications. For efficient resolution of peptide mixtures, particularly those containing peptides with multiple positive charges, our results suggest that 0.2–0.25% TFA in the mobile phase will achieve the desired goal of optimum peak resolution and detection sensitivity. In addition, the use of high temperature as a complement to such levels of TFA concentration has also proved effective in maximizing peptide resolution.

Acknowledgements

This work was supported by an NIH grant (RO1 GM 61855) to Robert S. Hodges.

References

[1] C.T. Mant, R.S. Hodges (Eds.), High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation, CRC Press, Boca Raton, FL, 1991.
[2] R.L. Cunico, K.M. Gooding, T. Wehr (Eds.), HPLC and CE of Biomolecules, Bay Bioanalytical Laboratory, Richmond, 1998.
[3] C.T. Mant, R.S. Hodges, in: K.M. Gooding, F.E. Regnier (Eds.), HPLC of Biological Macromolecules, Marcel Dekker, New York, 2002, p. 433.
[4] D. Guo, C.T. Mant, R.S. Hodges, J. Chromatogr. 386 (1987) 205.
[5] F.E. Regnier, Methods Enzymol. 91 (1983) 137.
[6] C.T. Mant, R.S. Hodges, Chromatographia 24 (1987) 805.
[7] Cs. Horváth, W. Melander, I. Molnár, J. Chromatogr. 125 (1976) 129.
[8] Cs. Horváth, W. Melander, I. Molnár, P. Molnár, Anal. Chem. 49 (1977) 2295.
[9] P.T. Kissinger, Anal. Chem. 49 (1977) 883.
[10] J.C. Kraak, K.M. Jonker, J.F.K. Huber, J. Chromatogr. 142 (1977) 671.
[11] J.L.M. van de Venne, J.L.H.M. Hendrikx, R.S. Deedler, J. Chromatogr. 167 (1978) 1.
[12] A. Hong, M.M. Brasseur, D. Kesuma, in: C.T. Mant, R.S. Hodges (Eds.), High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis and Conformation, CRC Press, Boca Raton, FL, 1991, p. 765.
[13] D. Guo, C.T. Mant, A.K. Taneja, J.M.R. Parker, R.S. Hodges, J. Chromatogr. 359 (1986) 519.
[14] J.L. Glajch, J.J. Kirkland, J. Köhler, J. Chromatogr. 384 (1987) 81.
[15] J.J. Kirkland, J.L. Glajch, R.D. Farlee, Anal. Chem. 61 (1989) 2.
[16] J.L. Glajch, J.J. Kirkland, LC·GC 8 (1990) 140.
[17] B.E. Boyes, J.J. Kirkland, Pept. Res. 6 (1993) 249.
[18] Y. Chen, C.T. Mant, R.S. Hodges, J. Pept. Res. 59 (2002) 18.
[19] N.E. Zhou, O.D. Monera, C.M. Kay, R.S. Hodges, Protein Pept. Lett. 1 (1994) 114.
[20] O.D. Monera, T.J. Sereda, N.E. Zhou, C.M. Kay, R.S. Hodges, J. Pept. Sci. 1 (1995) 319.
[21] T.J. Sereda, C.T. Mant, R.S. Hodges, J. Chromatogr. A 695 (1995) 205.
[22] Y. Chen, C.T. Mant, R.S. Hodges, J. Chromatogr. A 1010 (2003) 45.
[23] W.S. Hancock, R.C. Chloupek, J.J. Kirkland, L.R. Snyder, J. Chromatogr. A 686 (1994) 31.
[24] R.C. Chloupek, W.S. Hancock, B.A. Marchylo, J.J. Kirkland, B.E. Boyes, L.R. Snyder, J. Chromatogr. A 686 (1994) 45.
[25] C.T. Mant, L.H. Kondejewski, P.J. Cachia, O.D. Monera, R.S. Hodges, Methods Enzymol. 289 (1997) 426.
[26] C.T. Mant, Y. Chen, R.S. Hodges, J. Chromatogr. A 1009 (2003) 29.
[27] C.T. Mant, B. Tripet, R.S. Hodges, J. Chromatogr. A 1009 (2003) 45.
[28] F.D. Antia, Cs. Horváth, J. Chromatogr. 435 (1988) 1.
[29] J.W. Li, P.W. Carr, Anal. Chem. 69 (1997) 2202.
[30] J.W. Li, P.W. Carr, Anal. Chem. 69 (1997) 2550.
[31] N.E. Zhou, C.T. Mant, R.S. Hodges, Pept. Res. 3 (1990) 8.