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Potential of silica monolithic columns in peptide separations

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Abstract

The objective of the work described here was to evaluate the efficacy of silica monolith supports in high-speed reversed-phase liquid chromatography (RPLC) of peptides. This was done using a commercial Chromolith column with an octadecylsilane stationary phase and a tryptic digest of cytochrome *c*. Columns (100 mm × 4.6 mm) were operated at mobile phase velocities ranging from 1 ml/min (2.0 mm/s) to 10 ml/min (25 mm/s). There was little noticeable change over this flow rate range in either resolution, peak elution volume, or analyte concentration in collected fractions. It was concluded that capillary columns in this silica monolith format would be particularly valuable in peptide separations for proteomics. There was, however, a small, but perceptible contamination of peaks at high mobile phase velocity with earlier eluting analytes. Based on the fact that peak shape did not change at high mobile phase velocity, it is suggested that this phenomena might be due to the presence of peptide conformers in structural equilibrium on the sorbent surface. When elution rate exceeds the rate of conformer interchange, conformers could elute as broadened or even separate peaks.

Keywords: Monolithic columns; Peptides; Cytochromes

1. Introduction

There is increasing dependence in proteomics on reversed-phase liquid chromatography (RPLC) for the resolution of complex peptide mixtures. After initial application of RPLC to peptide separations in 1976 [1], the technique went through a stage of rapid development and expanded use in the decade that followed. Pivotal in the acceptance of RPLC for peptide separations was the discovery of (1) ion-paring agents that both increased peptide recovery and selectivity [2]; (2) high-porosity, high-purity silica supports that increased resolution and recovery [3]; (3) the influence of different alkylsilanes and surface derivatization protocols on selectivity and column longevity; and (4) optimization strategies for the separation of complex mixtures [4]. The current need in proteomics for even higher resolution and throughput has rekindled interested in advancing RPLC of peptides.

Among the technologies now being examined are the use of new micron-size porous and non-porous packing materials [5], ultrahigh-pressure columns of a meter in length [6,7], capillary electrochromatography (CEC) [8], open tubular columns [9], various kinds of multidimensional chromatography [10], microfabricated columns [11], and monolithic separation media [12]. Most of these approaches are directed more at increasing resolution than separation speed. Separation speed is limited in porous media by stagnant mobile phase mass transfer [13], i.e. the rate of analyte transfer between the mobile phase passing through the interstitial space between sorbent particles and stagnant pools of liquid in support pore matrices. A number of solutions to this problem are currently under examination, including elimination of support pores, the use of very small particles, and recently the use of silica monolith columns to enhance mass transfer [14].

Silica monolith fabrication has been achieved in several ways [15–18]. One is through a sol–gel approach involving hydrolysis and polycondensation of precursors such as tetramethoxysilane to produce a continuous bed of porous silica. The resulting silica-based gel network offers high permeability, reasonable surface area, good heat stability, and excellent resistance to solvents. An alkyl silane stationary phase is applied after matrix fabrication to obtain a reversed-phase column. The protocol for producing what seems to be the most successful silica monolith columns comes from Tanaka and co-workers [19,20]. The heart of their protocol is hydrolytic polymerization of tetramethoxysilane accompanied by phase separation in the presence of pore forming, water-soluble organic polymers. Monolith columns have also been prepared by entrapping preformed support particles in the silica sol–gel. Although

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stable, durable columns have been produced by this approach [21–25], their potential and availability as commercial columns seems far more limited than continuous rod columns.

The focus in this paper is on determining the efficacy of a commercial silica monolith column for the rapid separation of peptides as would be needed in proteomics. Commercial "Chromolith" columns were evaluated with a tryptic digest of bovine cytochrome *c* to determine the impact of separation time on column efficiency, analyte recovery, and analyte purity. Analyte purity was determined using matrix-assisted laser desorption ionization (MALDI) mass spectrometry.

2. Experimental

2.1. Materials

Bovine cytochrome *c*, tris(hydroxymethyl)aminomethane (Tris base), tris(hydroxymethyl)aminomethyl hydrochloride (Tris acid), iodoacetic acid, cysteine, dithiothreitol (DTT), *N*-tosyl-L-lysine chloromethyl ketone (TLCK), and α-cyano-4-hydroxycinnamic acid were purchased from Sigma (St. Louis, MO, USA). Chromolith columns 100 mm × 4.6 mm were kindly provided by Merck Japan (Tokyo, Japan). Human andrenocorticotropic hormone (ACTH) fragment 18–39 and human angiotensin I were purchased from Bachem (Torrance, CA, USA), sequencing grade trypsin was purchased from Promega (Madison, WI, USA). HPLC-grade acetonitrile (ACN) was obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA). HPLC-grade trifluoroacetic acid (TFA) was purchased from Pierce (Rockford, IL, USA). All commercially available reagents were used directly without purification.

2.2. Proteolysis

Five milligrams of bovine cytochrome *c* were dissolved in 0.1 M Tris buffer containing 8 M urea, pH 8.0. DTT was then added to 10 mM final concentration to reduce disulfide bonds. After incubation at 37 °C for 2 h, iodoacetic acid was added to 20 mM in concentration and incubated on ice in darkness for another 2 h. The reaction was quenched with 10 mM cysteine for 30 min at room temperature to consume excess iodoacetic acid. After diluting to a final urea concentration of 0.8 M with 0.1 M Tris digestion buffer (pH 8.0) containing 10 mM $CaCl_2$, TPCK-treated trypsin was added (at roughly a 1:50 enzyme to protein molar ratio) and incubated at 37 °C for 24 h. Digestion was stopped by adding the protease inhibitor TLCK in molar excess to trypsin. The digest was then frozen in liquid N_2 until it was analyzed.

2.3. Chromatography

All chromatographic separations were performed with a Biocad Analytical Workstation from Applied Biosystems (Framingham, MA, USA). Bovine cytochrome *c* was prepared at a concentration of 1 mg/ml and tryptic digested for 24 h using a protein to enzyme ratio of 50:1. The resulting tryptic digest of bovine cytochrome *c* (200 μl) was applied to a 100 mm × 4.6 mm i.d. Chromolith C_{18} column (Merck, Japan) subsequent to equilibration with 5% ACN aqueous buffer containing 0.1% TFA. Peptides were eluted with a mobile phase gradient ranging from 5% ACN, 0.1% TFA in water to 60% ACN, 0.1% TFA in water. Eluted peptide peaks were collected for mass spectral analysis. Separation times of 60, 30, 15, 7.5, and 6.0 min were used at flow rates of 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 ml/min, respectively. Total elution volume was held constant in all cases. Fractions were manually collected and speed-dried before analysis by MALDI mass spectrometry.

2.4. MALDI mass spectrometry

MALDI mass spectrometry was performed by using a Voyager DE-RP BioSpectrometry Workstation from Applied Biosystems. One microliter of a saturated solution of α-cyano-4-hydroxycinnamic acid in ACN–water (50:50) containing 0.1% TFA was applied to the MALDI plate and allowed to dry before sample application. Peptides were analyzed in the reflector mode with delayed extraction. External calibration was achieved using a mixture of standard peptides containing angiotensin I (M_r 1296.68) and ACTH 18–39 (M_r 2465.70).

3. Results and discussion

High-resolution separations of peptides in the gradient elution mode often requires the passage of 60 or more column volumes of mobile phase. Using a common linear velocity of 2 mm/s; roughly 60 min would be required for the fractionation of a peptide mixture with a 100 mm long column. Depending on column efficiency, the elution time of individual peaks would be 30–45 s under these conditions. Based on the fact that modern mass spectrometers can acquire spectra in a second, a higher peak delivery rate could be accommodated by mass spectrometers used in proteomics if it were possible to do so without loss of chromatographic resolution. The objective of the work described below was to test the efficiency of a silica monolith RPLC column at increasing mobile phase velocity using the same gradient slope. Although gradient slope is generally described in terms of time at a given mobile phase velocity, it is less confusing and more accurate to define gradients in terms of total eluent volume or column volumes as was done here. The gradient volume used in the experiments described below was 60 ml in all cases. A 100 mm × 4.6 mm column was eluted with this gradient volume over the course of gradient times ranging from 6 to 60 min.

The separation of a tryptic digest of cytochrome *c* in 6 and 60 min is seen in Fig. 1. Even when the linear velocity

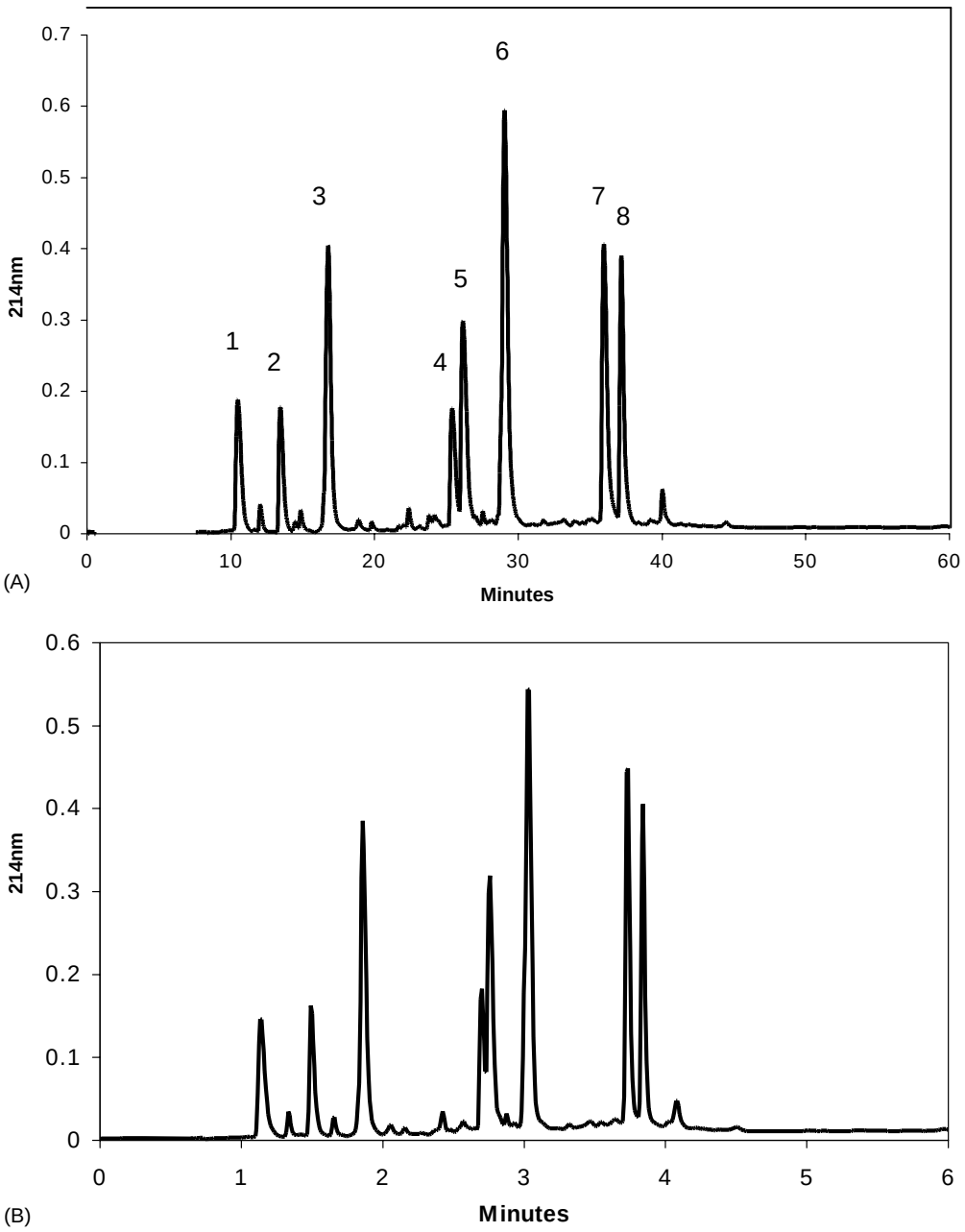

Fig. 1. Reversed-phase chromatograms of bovine cytochrome *c* tryptic digest at flow rates of (A) 1.0 ml/min with a 60 min gradient and (B) 10.0 ml/min with a 6 min gradient. Mobile phase composition ranged from 5 to 60% ACN in the presence of 0.1% TFA. Gradient volume was constant at 60 column volumes.

is 25 mm/s and the gradient time is 6 min at the highest mobile phase velocity, little degradation of efficiency was observed at high mobile phase velocity. Column performance was further evaluated by examining the resolution of peaks 7 and 8 at linear velocities ranging from 1 to 10 ml/min (Table 1). Resolution at 25 mm/s linear velocity was 77% of that at 2.5 mm/s. The fact that peptide separations could be achieved 10 times faster than with a conventional packed column with moderate loss in resolution could have a major impact on analytical throughput in proteomics. Moreover, column operating pressure was still in the range of 150 bar at 10 ml/min. This is well within the capacity of most commercial LC instruments.

A concern was that operating columns at very high mobile phase velocity could cause greater sample dilution. Based on absorbance at 214 nm, it is seen in Fig. 2 that analyte concentration is almost independent of mobile phase velocity. There will be no loss in sensitivity at high mobile phase velocity if gradient slope is altered proportionately.

Solvent consumption is another concern. Although solvent consumption per unit time is much higher at high mobile phase velocity, sample fractionation is achieved in

Table 1
Resolution of peaks 7 and 8 from Fig. 1 as a function of mobile phase velocity

Flow rate (ml/min)	Linear velocity (mm/s)	Δt_R, peaks 7 and 8 (min)	$\frac{1}{2}(W_7+W_8)$	Resolution[a]
1	2.5	1.209	0.275	4.40
2	5.0	0.584	0.158	3.70
4	10	0.300	0.081	3.70
8	20	0.133	0.0395	3.37
10	25	0.109	0.032	3.41

[a] Resolution of peaks 7 and 8 according to the equation $R_s = 2(\Delta t_R)/(W_{1,1} + W_{1,2})$.

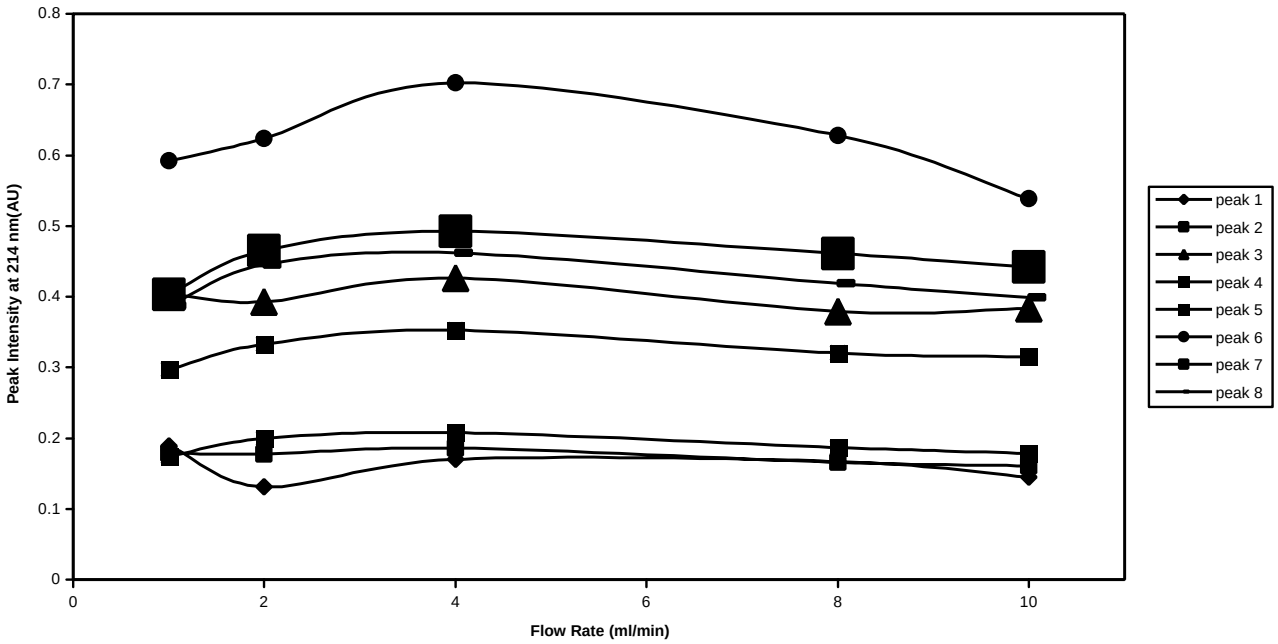

Fig. 2. Peak concentration as a function of mobile phase velocity. Analytes used in this study were derived from the tryptic digest of bovine cytochrome *c*. Analyte peak numbers and chromatography conditions are the same as in Fig. 1 with the exception of mobile phase velocity. Mobile phase velocities used in these experiments were 1.0, 2.0, 4.0, 8.0 and 10.0 ml/min.

a correspondingly shorter time and sample throughput is higher. This is possible because gradient volume is independent of mobile phase velocity.

Still another concern of operating columns at higher mobile phase velocity is that analyte purity will degrade. Analyte purity was monitored in these studies with MALDI mass spectrometry. Peptide fractions from the cytochrome *c* digest were manually collected and examined by MALDI-MS (Table 2). The peptides KTGQAPGFSYTDAN and GITWGEETLMEYLENPKK have "missed" trypsin cleavages resulting from the fact that cytochrome *c* has adjacent basic amino acids and initial cleavage can occur at either of these amino acids with equal probability. Once trypsin has cleaved a protein in such a manner that two basic amino acids are adjacent at either the N- or C-terminus of a peptide, there is a low probability trypsin will further cleave between K–K, R–K, R–R, or K–R residues because it is an endopeptidase. The largest and smallest peptides observed were an octadecapeptide (from residues 56 to 73) of M_r 2137.7 and pentapeptide of M_r 634.2 (from residues 9 to 13) at the amino terminus of the protein, respectively. Small hydrophilic peptides were so weakly retained by the Chromolith column that they eluted in the void volume and were not observed. This problem is not unique to this column, reversed-phase columns in gen-

Table 2
Tryptic digest mass fingerprinting of bovine cytochrome *c*

Peak no.	Mass	Position	No. MC	Peptide sequence
2	634.238	9–13	0	IFVQK
3	1456.450	40–53	0	TGQAPGFSYTDANK
	1584.535	39–53	1	KTGQAPGFSYTDAN
4	779.377	80–86	0	MIFAGIK
	1296.641	28–39	1	TGPNLHGLFGRK
5	1168.576	28–38	0	TGPNLHGLFGR
6	1633.584	9–22	1	IFVQKCAQCHTVEK
7	2137.700	56–73	1	GITWGEETLMEYLENPKK
8	2009.412	56–72	0	GITWGEETLMEYLENPK

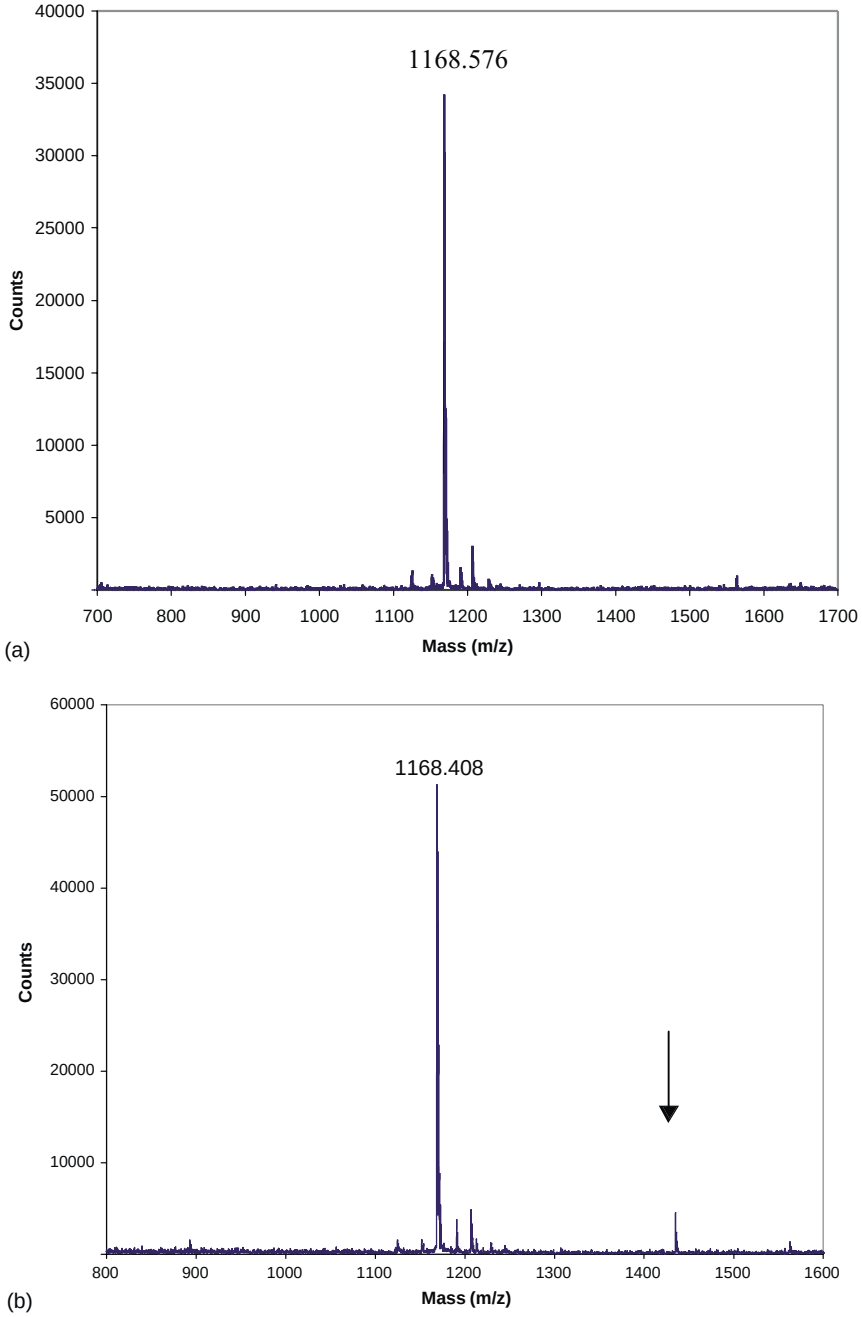

Fig. 3. MALDI mass spectrometry of peak 5 collected at a flow rate of (a) 1.0 ml/min and (b) 10.0 ml/min using α-cyano-4-hydroxycinnamic acid in ACN–water (50:50) containing 0.1% TFA as the matrix.

eral do not retain small hydrophilic peptides. The spectra for TGPNLHGLFGR from peak 5 at 1 and 10 ml/min are virtually identical with the exception of a small additional peak in the spectrum at *m*/*z* 1456 (Fig. 3). The same is true for the peptide IFVQKCAQCHTVEK in peak 6, except that an impurity is seen at *m*/*z* 1168 (Fig. 4). This peak is obviously from TGPNLHGLFGR that eluted in peak 5, just before this fraction. The same phenomenon is seen in the spectra of GITWGEETLMEYLENPK from peak 8 at mobile phase velocities of 1 and 10 ml/min (Fig. 5). The amount of impurity from GITWGEETLMEYLENPKK in peak 7 is larger at 10 ml/min. Although the chromatograms in Fig. 1 clearly show peak resolution, impurities from earlier eluting fractions are seen in the spectra of later fractions (Figs. 3–5). This phenomenon could be due to manual collection errors, but it probably is not. It is much more likely that at very high mobile phase velocity and rapid gradient generation some kinetic effect is responsible for this phenomenon. Peptide analytes may be adsorbed to a hydrophobic surface in multiple conformations [26]. It is

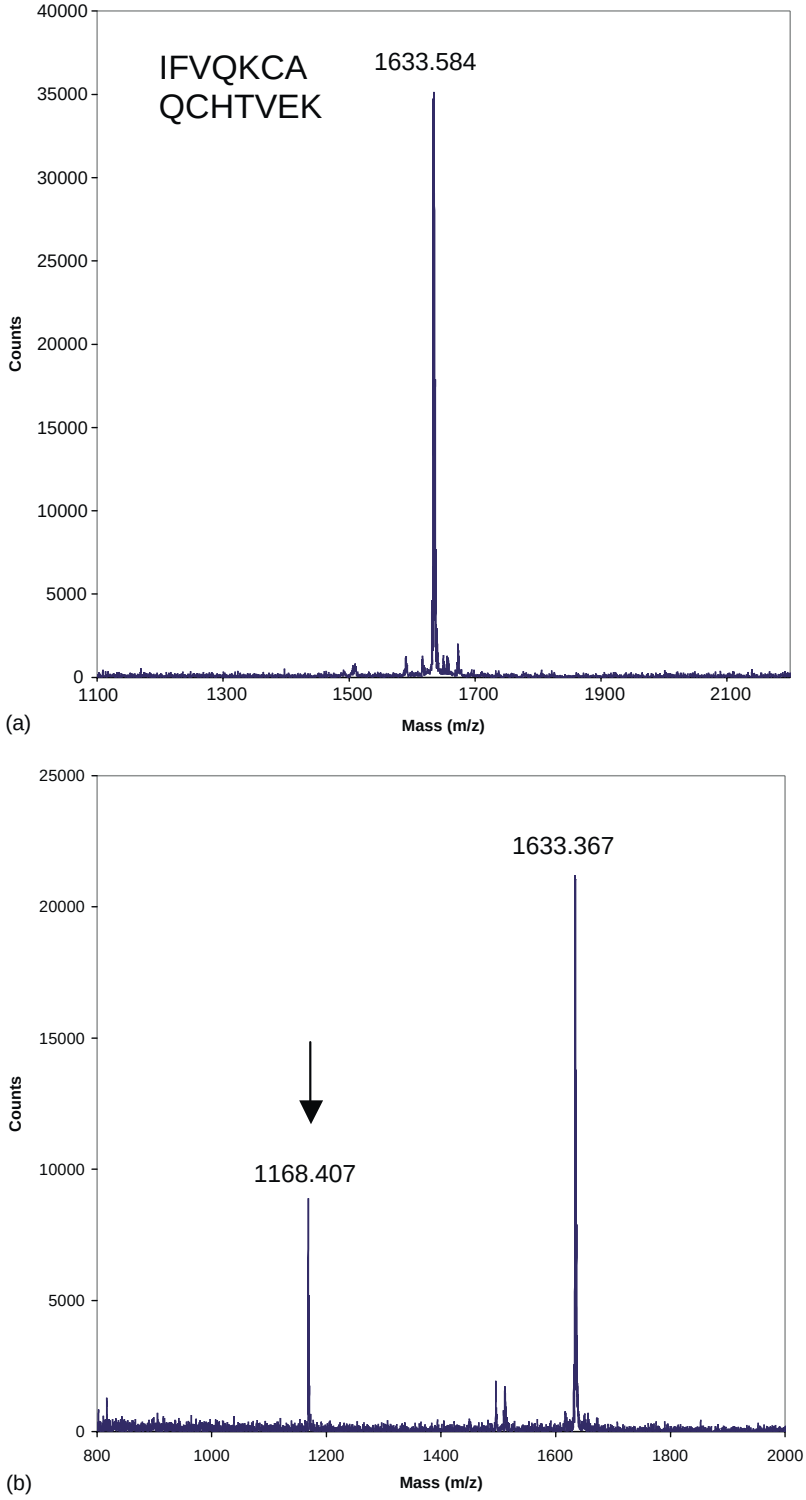

Fig. 4. MALDI mass spectrometry of peak 6 collected at a flow rate of (a) 1.0 ml/min and (b) 10.0 ml/min with α-cyano-4-hydroxycinnamic acid in ACN–water (50:50) containing 0.1% TFA as the matrix.

possible that some conformers desorb more rapidly than others [27]. If the rate of desorption and elution is shorter than the rate at which structural isoforms equilibrate on the sorbent surface, a phenomenon such as that observed in Figs. 3–5 would be observed. The tailing of peaks into later eluting fractions appears not to be unique to these experiments. Personal communication with others working in the field of proteomics indicate that this phenomenon occurs in cation exchange separations of peptides as well, even at mobile phase velocities of 2 mm/s. This is not a

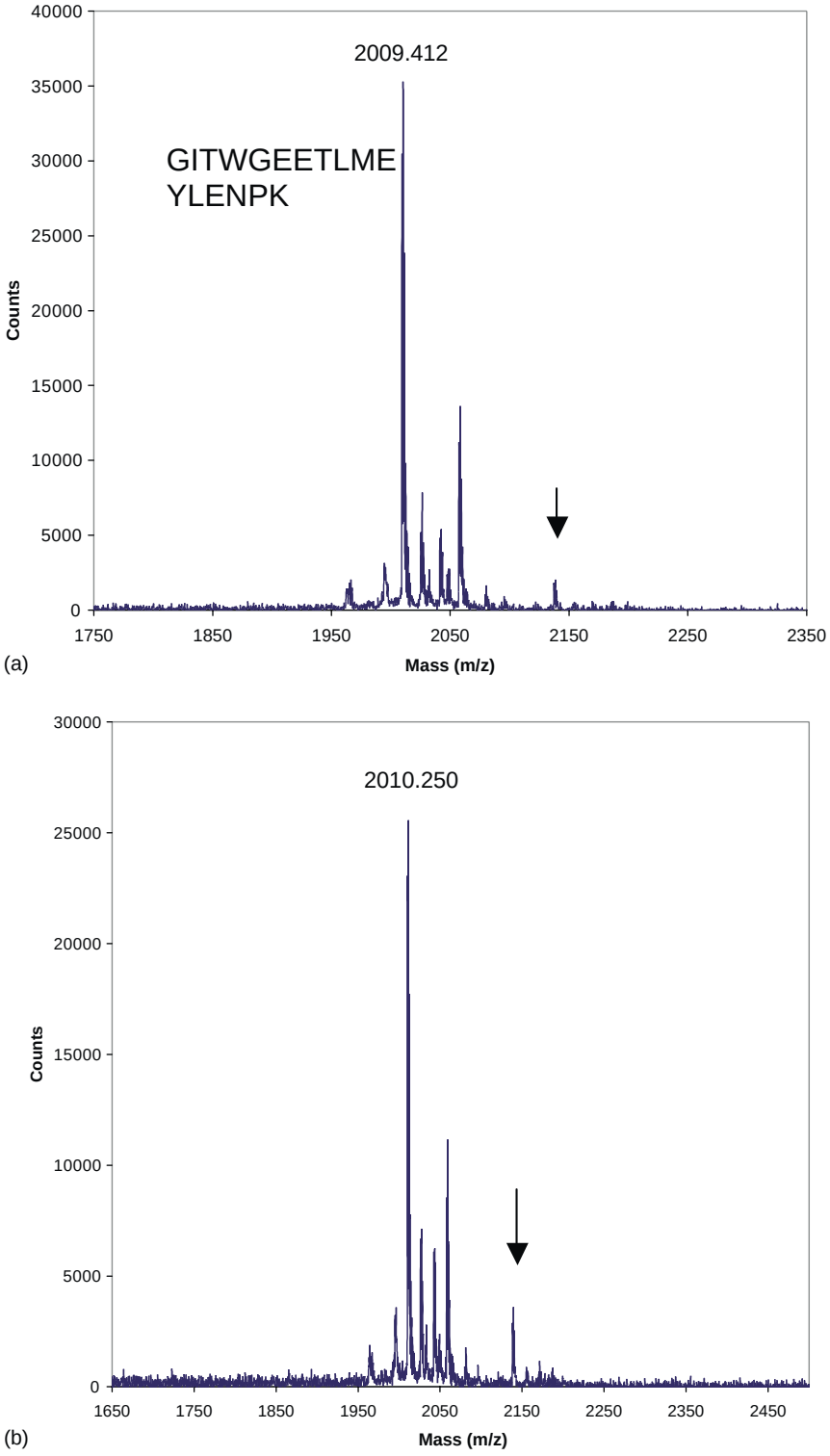

Fig. 5. MALDI mass spectrometry of peak 7 collected at a flow rate of (a) 1.0 ml/min and (b) 10.0 ml/min with α-cyano-4-hydroxycinnamic acid in ACN–water (50:50) containing 0.1% TFA as the matrix.

serious problem unless samples are very complex and all the separation space in a mass spectrum is needed. For example, it is common in the analysis of tryptic digests of a proteome that fractions from a reversed-phase chromatography column can contain one hundred or more peptides. The appearance of large numbers of abundant peptides from an earlier fraction can obscure low abundance peptides in the mass spectrum of a later fraction. Moreover, failure of a peak to elute as a single, sharp peak can be a serious problem in quantification.

4. Conclusion

It may be concluded from the data presented here that silica monolith reversed-phase chromatography columns show little loss in the resolution of peptides ranging up to several thousand in molecular weight as mobile phase velocity is elevated from the conventional 2.5–25 mm/s. Moreover, at 25 mm/s with a 100 mm length column, operating pressure did not exceed 150 bar. Pressures of this magnitude are easily obtained with conventional high performance liquid chromatography equipment. The only negative feature of very fast gradient elution noted during these studies was that some analytes carry over into later fractions. Because there was no noticeable change in either peak shape or analyte concentration, it is concluded that a small amount of analyte is more slowly desorbed in very rapid separations and elutes later. Whether this phenomenon is intrinsic to rapid chromatography or can be managed through either mobile phase or stationary phase selection remains to be determined.

It is further concluded that silica monolith chromatography columns will be of great value in the analysis of peptides. When the objective is to determine the relative concentration of peptide analytes with absorbance detectors and sample volume is not critical, columns of 4.6 mm internal diameter are suitable. In contrast, columns of this diameter will be of far less utility when the objective is to prefractionate a sample before it is introduced into a mass spectrometer through an electrospray ionization (ESI) interface. ESI interfaces cannot accommodate flow rates of 10 ml/min without splitting away a major portion of the column effluent. Column diameter must be reduced at least 10-fold for operation at linear velocities of 25 mm/s or more. Even at 1 ml/min, it is necessary to split the eluent stream entering an ESI interface.

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