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Application Update 183

Separation of Peptides from Enzymatic Digestion on Different Acclaim Columns: A Comparative Study

INTRODUCTION

Separation of peptides—which can originate from protein enzymatic digestion or peptide synthesis—is of interest in modern biological research. High-resolution and high-peak capacity are the primary concerns in peptide mapping and general proteomics. In purification of synthetic peptides or separation of peptides with post-translational modifications (PTM), however, both column selectivity and efficiency are needed to discriminate minor structural differences.

While possible protein conformation changes in organic solvents (i.e., denaturation) limit reversed-phase chromatography for most protein separations,[1] this concern does not apply to peptides, making reversed-phase chromatography the first choice for peptide separations. To achieve better peptide separations, more information is needed on selectivity and resolution of different reversed-phase columns and how the separation will be affected by separation parameters such as pH, ionic strength, etc.

A protein digest—a mixture of peptides that differ in hydrophobicity, charge, and size—provides a good test sample to understand a column's separation character (i.e., selectivity). In proteomics applications, a reversed-phase-based peptides separation provides a necessary preliminary step before mass spectrometry (MS), especially for complicated protein mixture digests.[2]

Thermo Scientific Acclaim™ 300 C18 columns are designed to provide excellent resolution and capacity for peptide mapping,[3] synthetic peptide separations,[4] and small protein separations. The Acclaim RSLC 120 C18 columns are designed for fast separation without sacrifice in resolution. The Acclaim RSLC PolarAdvantage II (PA2) columns feature amide-embedded chemistry with selectivity complementary to RSLC C18 columns, especially for polar peptides. This specially designed column can work with 100% aqueous mobile phase and has a wide pH stability range from 1.5–10. Both features can be useful for separating peptides.

In the work shown here, a tryptic digest of myoglobin was separated on these three Acclaim columns. Resolution and selectivity of the three columns were compared and major peaks in the myoglobin digest were identified using MS data. The effect of pH on peptide retention time was evaluated with respect to the peptides' characteristics (e.g., hydrophobicity, charge). The Acclaim PA2 column's two prominent features—compatibility with 100% aqueous and high pH mobile phases—were demonstrated to be useful for separating peptides. All separations were performed on the Thermo Scientific Dionex UltiMate™ 3000 RSLC system with diode-array detection (DAD).

EQUIPMENT

UltiMate 3400 RSLC system including:

- LPG 3400RS Pump
- WPS 3000TRS Autosampler
- TCC-3000 Thermostatted Column Compartment
- DAD-3000RS Diode Array Detector

Thermo Scientific MSQ™ Plus Single Quadrupole Mass Detector with an electrospray ionization (ESI) source

Thermo Scientific Dionex Chromeleon™ 6.80, SR9 Chromatography Workstation

REAGENTS AND STANDARDS

Deionized (DI) water, Gradient A 10, Milli-Q®

Acetonitrile (CH_3CN) HPLC grade, (Cat.# AC6100-0040) Fisher Chemical

Formic acid, 98%, Fluka

Ammonium formate, 99%, Acros Organics

Myoglobin from equine heart, ≥ 90% (Sigma P/N M1882)

Trypsin from bovine pancreas, TPCK Treated (P/N T1426)

DL-Dithiothreitol, ≥ 99% (Fluka P/N 43819)

Iodoacetamide, ≥ 99% (Sigma P/N I6125)

CHROMATOGRAPHIC CONDITIONS

Analytical Columns: Acclaim RSLC 120, C18, 2.2 µm, 2.1 × 100 mm (P/N 068982)
Acclaim 300, C18, 3 µm, 2.1 × 150 mm (P/N 060264
Acclaim RSLC PolarAdvantage II (PA2), 2.2 µm, 2.1 × 100 mm (P/N 068990)

Mobile Phase: (A) 2% acetonitrile in water
(B) 2% water in acetonitrile
(C) 10 mM formic acid
(D) 10 mM ammonium formate

Flow Rate: 0.42 or 0.2 mL/min

Inj. Volume: 2 µL

Column Temp.: 30 °C

UV Detection: Absorbance at 214 nm

Gradient for Figure 1 at pH 2.9: (Flow Rate as 0.2 mL/min)

Time (min)	A%	B%	C%	D%
0	78	2	19	1
55	30	50	19	1

Gradient for Figures 2, 3 at pH 2.9: (Flow Rate as 0.42 mL/min)

Time (min)	A%	B%	C%	D%
0	78	2	19	1
22	40	40	19	1
28	0	80	19	1

For gradients at other pH values in Figures 1–3, simply change the ratio C/D to 10/10 and 1/19. Mobile phase and gradient methods for Figures 4 and 5 were similar to those in Figures 2 and 3 with slight modifications as indicated in the figures.

MSQ-PLUS MASS DETECTOR CONDITIONS

Ionization Mode: ESI

Operating Mode: Positive Scan

Probe Temperature: 400 °C

Needle Voltage: 3.5 kV

Mass Range: 400~2000 amu

Scan Time: 0.5 sec

Cone Voltage: 50 V

Nebulizer Gas: Nitrogen at 75 psi

PREPARATION OF SOLUTIONS

Ammonium Bicarbonate, 50 mM

Dissolve 0.395 g of ammonium bicarbonate in 90 mL DI water and bring the volume to 100 mL with DI water.

Urea Solution, 8 M

Dissolve 0.48 g of urea in 0.6 mL ammonium bicarbonate solution and bring the volume to 1 mL with ammonium bicarbonate solution.

Dithiothreitol (DTT) Solution, 0.5 M

Dissolve 7.7 mg DTT in 100 µL ammonium bicarbonate solution. Prepare this solution immediately before use.

Iodoacetamide Solution, 0.5 M

Dissolve 18.5 mg iodoacetamide in 100 µL ammonium bicarbonate solution. Prepare this solution immediately before use.

PROTEIN DIGESTION PROCEDURE

Reduction and Alkylation

1. Weigh 5 mg of myoglobin in a microcentrifuge tube and add 1 mL 8 M urea solution. Gently mix to dissolve.
2. Add 20 µL of 0.5 M DTT to the microcentrifuge tube, followed by gentle mixing. Incubate for 60 min at 56 °C.
3. Cool the sample to room temperature.
4. Add 40 µL of 0.5 M iodoacetamide to the mixture. Gently mix and incubate in the dark for 30 min at room temperature.
5. Add another 80 µL of 0.5 M DTT to the mixture. Gently mix and incubate for 30 min at room temperature.

Dialysis of Myoglobin Sample

Dialyze (3.5 kD molecular weight [MW] cut-off) the reduced and alkylated myoglobin sample against 2 L of 50 mM ammonium formate for 24 h at room temperature. Change the dialysis buffer once during the 24 h period.

Trypsin Digest

Dissolve 2 mg trypsin in 200 µL ammonium bicarbonate solution. Add 20 µL trypsin solution (1 µg/µL) to the protein mixture. Gently mix and incubate for 20 h at 37 °C. Boil the mixture for 15 min to stop the reaction.

RESULTS AND DISCUSSION

Identified Peptides with Predicted MW

Table 1 shows the expected 18 myoglobin peptides, with no miscleavage, after tryptic digestion; among them are the 14 peptides that were identified by MS. Note: Peptide fragmentation by tandem mass spectrometry is commonly used to identify peptides in peptide mapping experiments. Table 2 shows identified peptides in Figures 1–3.

The MSQ Plus Mass Detector does not have tandem MS capability; therefore, the authors used the intact peptides' mass comparison with experimental tryptic digestion data for identification. For this study with the focus on LC separation and only a single protein tryptic digest (myoglobin), this approach can be used with confidence.

Table 1. Expected and Detected Myoglobin Tryptic Peptides

No	Range	Mn	z	MC#	Sequence
1	[1–16]	1946.9	1	0	GLSDGEWQQVLNVWGK
2	[17–31]	1606.8	1	0	**VEADIAGHGQEVLIR**
3	[32–42]	1271.7	1	0	**LFTGHPETLEK**
4	[43–45]	409.2	1	0	**FDK**
5	[46–47]	294.2	1	0	**FK**
6	[48–50]	397.3	1	0	**HLK**
7	[51–56]	708.3	1	0	**TEAEMK**
8	[57–62]	662.3	1	0	ASEDLK
9	[63–63]	147.1	1	0	K
10	[64–77]	1378.8	1	0	**HGTVVLTALGGILK**
11	[78–78]	147.1	1	0	K
12	[79–79]	147.1	1	0	K
13	[80–96]	1854.0	1	0	GHHEAELKPLAQSHATK
14	[97–98]	284.2	1	0	**HK**
15	[99–102]	470.3	1	0	**IPIK**
16	[103–118]	1885.0	1	0	**YLEFISDAIIHVLHSK**
17	[119–133]	1502.7	1	0	**HPGDFGADAQGAMTK**
18	[134–139]	748.4	1	0	**ALELFR**
19	[140–145]	631.3	1	0	**NDIAAK**
20	[146–147]	310.2	1	0	**YK**
21	[148–153]	650.3	1	0	**ELGFQG**

Sequences with no miscleavage, noted in bold, were observed in the experiment.
MC# stands for miscleavage number. Sequences (FDKFK, ASEDLKK, KK, HKIPIK, and KGHHEAELKPLAQSHATK) with a miscleavage were observed as well.

In addition to the correctly cleaved peptides, four peptides with one miscleavage were also observed by MS. Multiple properties affect a tryptic peptide's detectability by MS, as 50–90% sequence coverage has been reported in the literature.[5] Together with the coverage of the peptides with a miscleavage, approximately 90% coverage was achieved in this experiment.

Table 2. Identified Peptides in Figures 1–3				
Peak No.	**Expected *m/z***	**Observed *m/z***	**Range**	**Sequence**
2 (single charged)	470.3	470.3	99–102	IPIK
3 (single charged)	650.3	650.2	148–153	ELGFQG
5 (single, double charged)	684.4/342.7	684.4/342.7	43–47	FDKFK
7 (double charged)	1502.7/751.8	751.8	119–133	HPGDFGADAQGAMTK
9 (double charged)	735.5/368.2	368.2	97–102	HKIPIK
12 (double, triple charged)	1271.7/636.6/424.5	636.6/424.5	32–42	LFTGHPETLEK
13 (double, triple charged)	1606.8/803.9/536.3	803.9/536.3	17–31	VEADIAGHGQEVLIR
16 (single charged)	748.4	747.8	134–139	ALELFR
17 (triple charged)	1982.0/661.3	661.2	79–96	KGHHEAELKPLAQSHATK

Comparison of the Separation of Myoglobin Tryptic Peptides on Three Acclaim Columns at Different pH Values

As shown in Figure 1, the Acclaim 300 C18 column delivers a good separation of myoglobin tryptic peptides. When mobile phase pH changes from 5.6 to 2.9, peak width is slightly broadened, but the more acidic condition produces a better separation. For example, peaks 6, 8, and 3 are separated at pH 2.9 and they are not baseline resolved at pH 5.6. Peaks 15 and 16 are eluted as one peak at pH 5.6 and well separated at pH 2.9. Better separations and more sensitive MS signal at pH 2.9 confirm that acidic pHs (pH < 3) are preferred for reversed-phase high-performance liquid chromatography (RP-HPLC) peptide mapping.

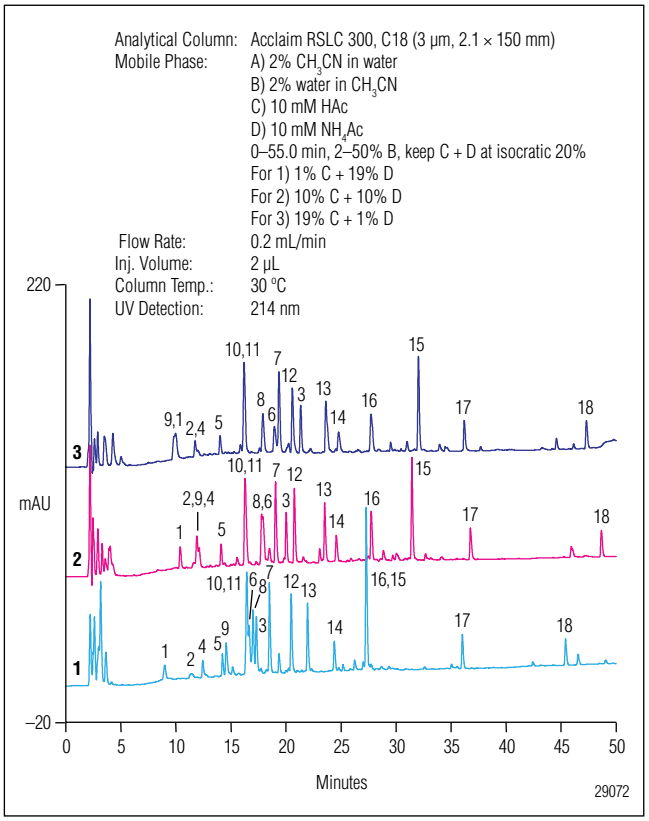

Figure 1. Peptide mapping of myoglobin tryptic digest on the Acclaim 300 C18 column at pH 5.6 (1), pH 3.7 (2), and pH 2.9 (3).

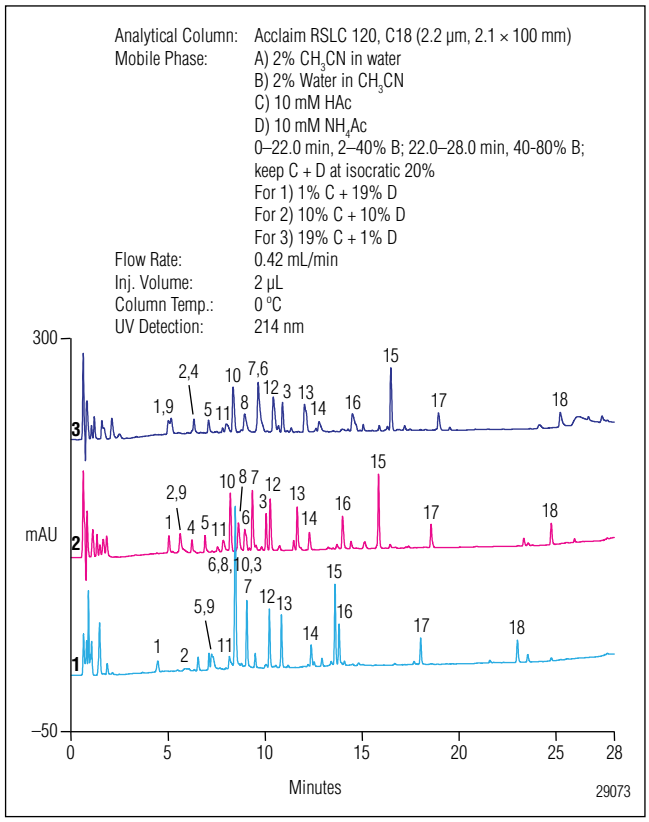

Figure 2 Peptide mapping of a myoglobin tryptic digest on the Acclaim RSLC C18 column at pH 5.6(1), pH 3.7 (2) and pH 2.9 (3).

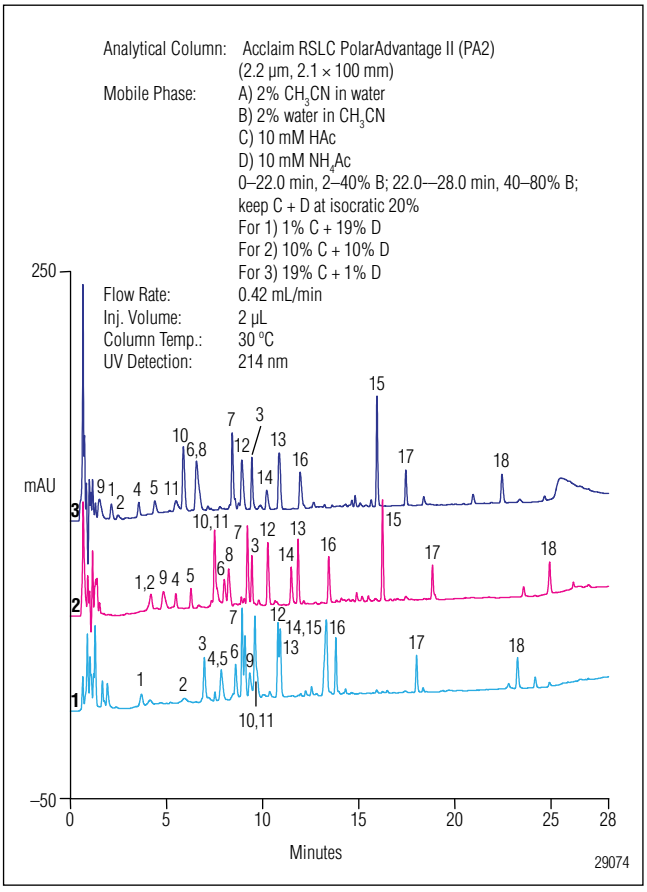

Figure 3. Peptide mapping of myoglobin on the Acclaim RSLC PA2 column at pH 5.6 (1), pH 3.7 (2) and pH 2.9 (3).

This does not appear to be the case with the Acclaim 120 C18 column. As Figure 2 shows, the Acclaim RSLC C18 column achieves very good resolution of the myoglobin peptides, especially at pH 3.7. Almost all peaks are baseline resolved at pH 3.7. At pH 2.9, three pairs of peaks (peaks 1 and 9, peaks 2 and 4, and peaks 7 and 6) coelute. At pH 5.6, peaks 6, 8, 10, and 3 coelute.

These data show that the pH of the mobile phase is important to retention time of peptides, and simply changing pH can be a good solution to resolve peaks that coelute under other conditions. Figure 3 shows the same pH experiment using the third column in this study, the Acclaim PA2 column in RSLC format. The best separations were achieved at pH 2.9 and 3.7, while pH 5.6 was not suitable for separation.

Effects of pH on Myoglobin Tryptic Peptides Elution Order

Most tryptic peptides maintain the same elution order on the Acclaim 300 C18 column with change in pH. Peaks 9 and 3 are exceptions. Peak 9 elutes earlier with the most acidic mobile phase. This peptide is identified as HKIPIK with pI approximately 10.6. Considering that the pKa of the histidine residue in the sequence is about 6.0, changing mobile phase pH from above 5.6 to below 2.9 adds one more positively charged residue to the peptide, thus reducing its hydrophobicity. This can explain the earlier elution of peak 9 at lower pH. This reduced affinity for the stationary phase is also observed with the Acclaim 120 C18 and PA2 columns. The most dramatic retention time change of peak 9 is observed on the Acclaim PA2 column. In contrast, peak 3 elutes later in the most acidic mobile phase. Peak 3 is identified as ELGFQG with a pI of 3.3. When mobile phase pH moves from near 5.6 to 3.3, the net charge of the peptide changes to near 0 from –1. The loss of charge increases the peptide's hydrophobicity and thus its retention.

Comparison of Myoglobin Tryptic Peptides Elution Order on Different Columns

The peptides eluted in very similar order on the Acclaim 300 C18 and 120 C18 columns, regardless of mobile phase pH. In contrast, there was a considerable difference in the peptide elution order at pH 5.6 when comparing the Acclaim C18 columns and the Acclaim PA2 column. This shows that the Acclaim PA2 column's embedded amide group provides a complementary selectivity to the Acclaim C18 column. As mobile phase acidifies, less difference was observed in the peptides' elution order between the Acclaim PA2 and C18 columns.

Advantages of the Acclaim PA2 Column: Compatibility with 100% Aqueous Mobile Phase

The stationary phase design of a hydrophilic group between the hydrophobic alkyl chain and silica surface enables the Acclaim PA2 column to remain wetted even in 100% aqueous mobile phase. While the conventional Acclaim C18 column will rapidly lose its selectivity due to dewetting in 100% aqueous mobile phase, the Acclaim PA2 column yields reproducible analyte retention time. A fraction of tryptic digested peptides, especially small peptides, are quite hydrophilic.[6] Even under 98% aqueous mobile phase, these peptides eluted with almost no retention. This results in poor chromatography and MS signal suppression.

As shown in Figure 4, peaks 1–10 eluted within 2 min using 98% aqueous mobile phase, a typical starting condition for peptide mapping. While some of this poor retention may be due to the small pore size of the Acclaim PA2 column, using 100% aqueous mobile phase separates peaks 4–10. In the myoglobin tryptic digest, these separated peptides constitute about 40% of all identified peptides. Additionally, 100% aqueous mobile phase provides some separation of peaks 16 and 17, which are unresolved in 98% aqueous mobile phase.

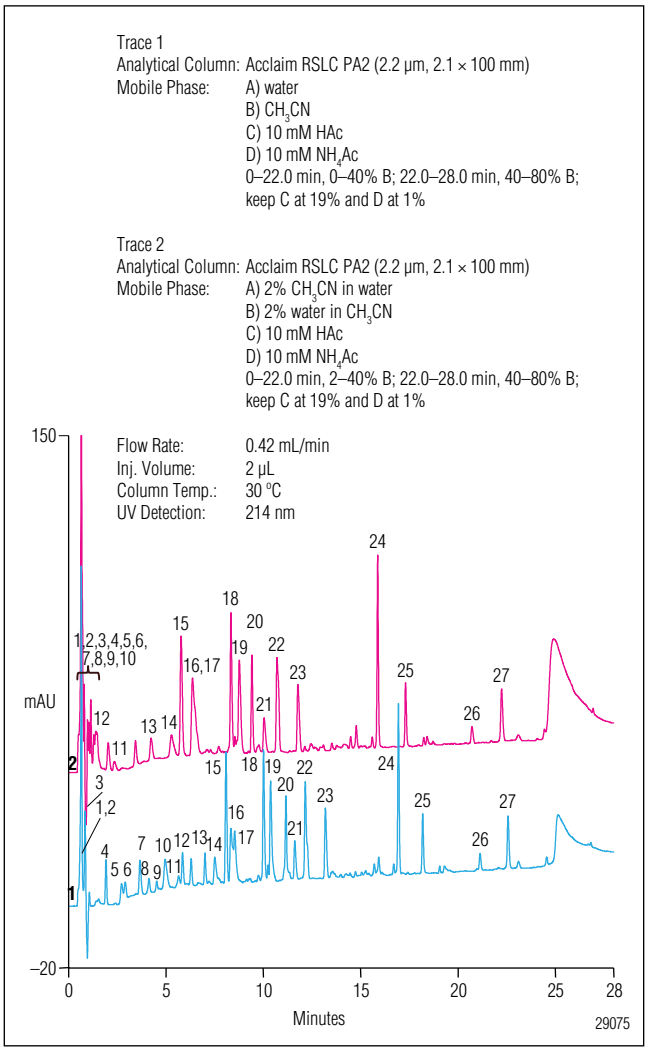

Figure 4. Peptide mapping of myoglobin on the Acclaim RSLC PA2 column with initial condition of mobile phase as (1) 100% water and (2) 2% CH_3CN in water.

Basic Mobile Phase on the Acclaim PA2 Column Provides Dramatically Different Selectivity

Routine peptide mapping involves separation of peptides by reversed-phase chromatography on a silica C18 column using an acidic mobile phase. Low pH usually provides high resolution, but separation with an alkaline mobile phase has been shown to be useful for peptides that are difficult to dissolve in acidic mobile phases and for some peptides that cannot be separated in acidic mobile phase.[7]

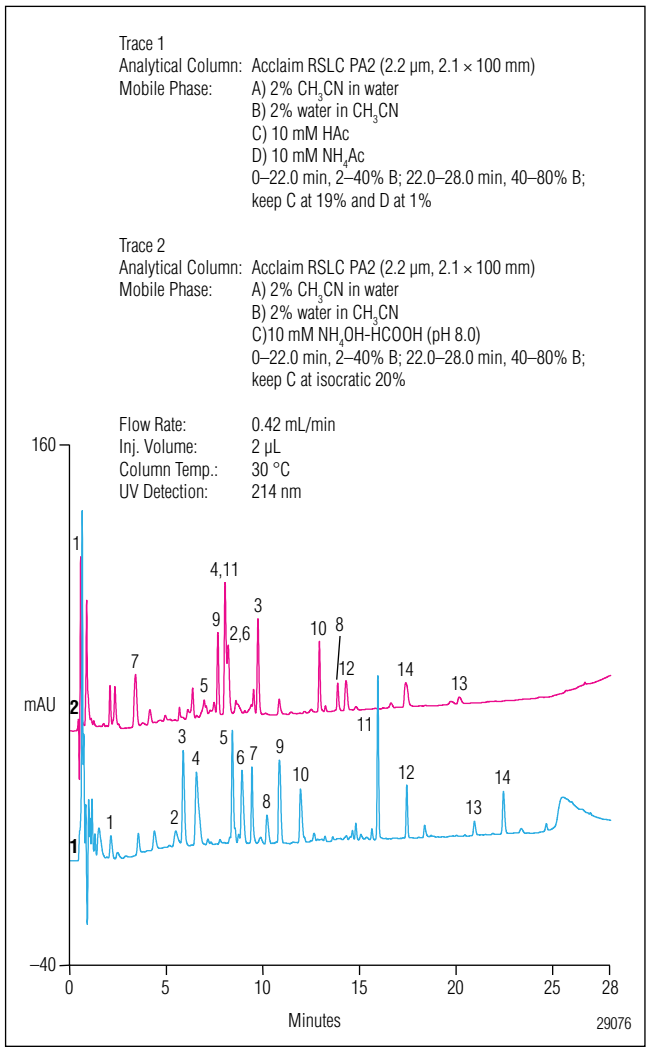

Figure 5. Peptide mapping of myoglobin on the Acclaim RSLC PA2 column at pH (1) 2.9, and (2) 8.0.

Conventional silica and most polar-embedded phases can not tolerate alkaline conditions. The Acclaim PA2 column is specially designed to withstand high pH conditions, and is highly stable in a wide pH range (1.5–10). Figure 5 shows peptide mapping on the Acclaim PA2 column at pHs 8.0 and pH 2.9. The elution order of the peptides is dramatically different. Although low pH separations can provide higher resolution, alkaline separation provides good resolution and complementary selectivity.

Peak 7, identified as peptide ELGFQG, is eluted much earlier at pH 8.0 than at pH 2.9. Due to its pI of 3.3, the peptide is fully charged at pH 8.0, whereas it is partially charged at pH 2.9. This explains the retention difference between the two pHs. Actually, most peaks elute earlier at pH 8.0 than at pH 2.9. This may be attributed to the embedded amide group in the Acclaim PA2 column. The amide will be partially charged under acidic conditions, which will contribute additional retention to polar analytes. The amide will lose its charged state at basic condition, which may decrease its retention of polar analytes.

Peak 10, peptide ALELFR, is an exception because it eluted earlier at pH 2.9 than at pH 8.0. With pI 7.0, peptide ALELFR can be almost noncharged at pH 8.0 and fully charged at pH 2.9. So the peptide is predicted to have longer retention time at pH 8.0.

CONCLUSION

This study showed the effect of pH on the separation of tryptic peptides on the Acclaim 300 C18, RSLC 120 C18, and RSLC PA2 columns. With the help of MS data, peptides were partially identified. Three types of columns achieved highest separation under different pHs. The elution of an individual peptide will be affected by charge status under different pHs. The compatibility with 100% aqueous mobile phase of the Acclaim PA2 column was shown to be advantageous for separating small polar peptides. The Acclaim PA2 column, with its featured hydrolytic stability under basic condition, demonstrated quite different selectivity with a basic mobile phase compared to routine acidic conditions. Both features are useful for peptide separations.

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