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The Influence of pH on Elution Order of Tryptic Peptides on Reversed-Phase Columns: A Unique Approach to Multidimensional Liquid Chromatography Separations for Proteomics

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Introduction

Recent developments in two-dimensional chromatography in which anion exchange is used first followed by reversed-phase in the second dimension coupled with mass spectrometry has already been utilized successfully to separate and detect peptides from yeast proteomes. However, this current method is limited in the first dimension by the low peak capacity offered by IEX columns and further requires a desalting step before introduction into the reversed-phase second dimension.

An evaluation of different reversed-phase stationary phases was carried out to determine and compare retention time, peak shape and peak capacity for peptide samples obtained from a tryptic digest of cytochrome c. Symmetry®, Spherisorb®, Resolve™, Nova-Pak® and XTerra® packings were all tested. The purpose of this experiment is to utilize this evaluation to develop a two-dimensional chromatography system where high pH reversed-phase separation is used first followed by low pH reversed-phase separation in the second dimension. The advantage of this technique would be to generate a salt-free, high capacity, high resolving separation technique in both dimensions that can readily be introduced into the mass spectrometer.

Experimental

Waters® Alliance® HPLC system
- –Gradient elution
- –Column temperature = ambient
- –Flow rate = 0.75 ml/min, split
  - 0.20 mL/min into mass spectrometer
  - 0.55 mL/min into PDA

Waters® 996 PDA
- –Detection at 214 nm

Waters® ZQ™ Mass Detector
- –Source = ESI (+)
- –Capillary (kV) = 3.5
- –Cone (V) = 30
- –Temperature (°C)
  - Source = 150
  - Desolvation = 350
- –Gas flow (L/Hr) ,Cone = 50, Desolvation = 550
- –Single Ion Recording (SIR) Mode
  - 14 Channels open
  - Corresponds to zero missed cleavage ions of Cytochrome C digested with trypsin.

Bovine Cytochrome C
- –Digested for 4 hours with trypsin at 37 °C

Columns Evaluated
- –XTerra® MS C_{18} 3.5µm 4.6 x 50 mm
- –XTerra® RP_{18} 3.5µm 4.6 x 50 mm
- –Symmetry® C_{18} 3.5µm 4.6 x 50 mm
- –Spherisorb® C_{18} 3 µm S3 ODS2 4.6 x 50 mm
- –Resolve™ C_{18} 5µm 4.6 x 50 mm
- –Nova-Pak® C_{18} 4µm 4.6 x 150 mm
- –Polarity™ dC_{18} 3.5µm 4.6 x 50 mm

CytocChrome C Bovine
Source: swissprot: locus CYC_BOVIN, accession P00006;
Average Mass = 11572.3752, Monoisotopic Mass = 11565.0207
N-Terminus = H, C-Terminus = OH
Digest: Trypsin:/K-\P /R-\P

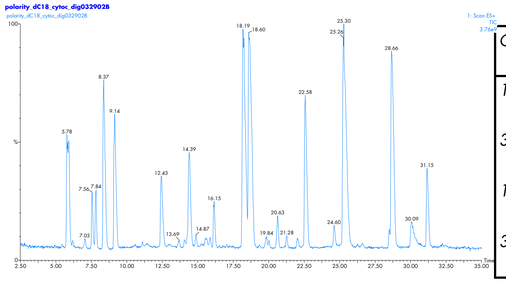
Frag#	Res#	Sequence	Theor(Bo)	[M+H]
T9	39-39	146.11	147.11	(R)K(T)
T13	73-73	146.11	147.11	(K)K(Y)
T20	100-100	146.11	147.11	(K)K(A)
T3	8-8	146.11	147.11	(K)K(I)
T16	87-87	146.11	147.11	(K)K(K)
T17	88-88	146.11	147.11	(K)K(G)
T2	6-7	203.13	204.13	(K)GK(K)
T6	23-25	260.15	261.16	(K)GGK(H)
T11	54-55	260.15	261.16	(K)NK(G)
T7	26-27	283.16	284.17	(K)HK(T)
T18	89-91	360.18	361.18	(K)GER(E)
T21	101-104	433.18	434.19	(K)ATNE(-)
T1	1-5	546.26	547.27	(-)GDVEK(G)
T4	9-13	633.38	634.39	(K)IFVQK(C)
T14	74-79	677.37	678.38	(K)YIPGTK(M)
T15	80-86	778.44	779.45	(K)MIFAGIK(K)
T19	92-99	963.53	964.54	(R)EDLIAYLK(K)
T5	14-22	1017.44	1018.45	(K)CAQCHTVEK(G)
T8	28-38	1167.61	1168.62	(K)TGPNLHGLFGR(K)
T10	40-53	1455.66	1456.67	(K)TGQAPGFSYTDANK(N)
T12	56-72	2008.95	2009.95	(K)GITWGEETLMEYLENPK(K)

Peak Capacity Values for Columns Tested under low pH Conditions

Columns (100 angstrom)	Peak Capacity
Polarity™ dC_{18}	276
Symmetry® C_{18}	205
XTerra® MSC_{18}	183
XTerra® RP_{18}	179

Peak Capacity = Time of Analysis (min)/ [S peak widths @ 50% height (min)/ number of peaks]

Total Ion Chromatogram of Low pH Separation of Cytochrome C Tryptic Digest on Polarity™ dC_{18} column



Effect of Pore Size on the Peak Capacity of Tryptic BSA Peptide Separations.

Column	Peak Capacity
100 Angstrom Column X	269
300 Angstrom Column X	164
100 Angstrom Column y	217
300 Angstrom Column y	102

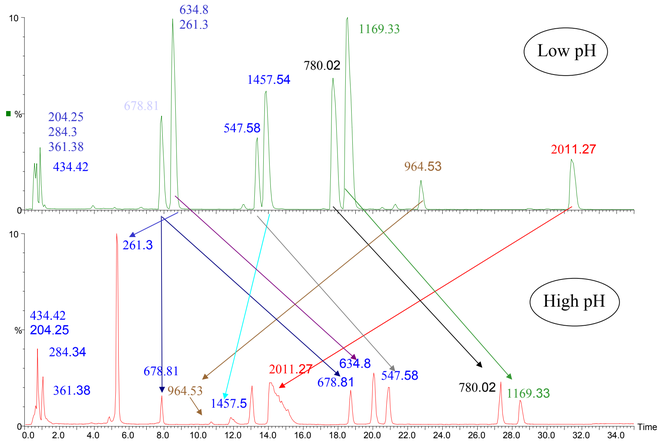
Retention Time (min) of Cytochrome C Tryptic Digest on Reversed-Phase Chemistries at Low pH Detected by MS

m/z	Sequence No.	XTerra® MSC_{18}	XTerra® RP_{18}	Polarity™ dC_{18}	Symmetry® C_{18}	Spherisorb®	Nova-Pak®
204.25	6-7	0.6	0.6	0.8	0.8	1.9	2.2
261.3	54-55	7.1	5.0	9.7	0.8	9.7	2.1
261.3	23-25	7.1	5.0	9.7	0.8	1.9, 2.4, 2.9	2.1
284.3	26-27	0.5	0.6	0.9	0.6	2.6	2.1
361.4	98-91	0.6	0.8	0.9, 18.9, 23.2	0.8,7.09,21.3	6.2	2.7
434.4	101-104	0.8	0.8	1.5	17.8	42.7	2.5
547.6	1-5	11.7	10.3	12.8	16.6	17.6	10.8
634.8	9-13	7.1	5.0	9.7	7.0	11.5	37.8
678.8	74-79	6.7	5.1	8.9	6.2,11.7,18.0	35.6	47.8
780.0	80-86	16.0	16.2	18.8	16.2	33.2	Not detected
964.5	92-99	22.1	22.3	23.2	21.3	In wash	Not detected
1169.3	28-38	16.4	17.0	19.2	16.6	Not detected	Not detected
1457.5	40-53	13.1	13.6	14.8	12.7	Not detected	Not detected
2011.27	56-72	30.8	29.7	31.7	29.4	In wash	Not detected

Retention Time (min) of Cytochrome C Tryptic Digest on XTerra® Columns at High and Low pH Detected by MS

m/z	Sequence No.	XTerra® MS C_{18} Low pH	XTerra® MS C_{18} High pH	XTerra® RP_{18} Low pH	XTerra® RP_{18} High pH
204.25	6-7	0.6	0.7	0.6	0.7
261.3	54-55	7.1	5.3	5.0	1.9
261.3	23-25	7.1	5.3	5.0	1.9
284.3	26-27	0.5	1.0	0.6	0.8
361.4	98-91	0.6	1.1	0.8	0.6
434.4	101-104	0.8	0.6	0.8	0.5
547.6	1-5	11.7	20.9	10.3	19.1
634.8	9-13	7.1	20.1	5.0	17.0
678.8	74-79	6.7	7.9, 13.0, 18.7	5.1	10.3, 11.9, 16.8
780.0	80-86	16.0	27.4	16.2	25.8
964.5	92-99	22.1	11.9	22.3	7.8
1169.3	28-38	16.4	28.5	17.0	27.9
1457.5	40-53	13.1	10.7	13.6	8.5
2011.27	56-72	30.8	14.2	29.7	10.6

Total Ion Chromatograph showing Peak Retention Time Movement with change in pH on XTerra® MS C_{18} 3.5 µm 4.6 x 50 mm



Low pH Separation Conditions
- A: H_2O
- B: MeCN
- C: 100 mM Formic Acid, pH 2.5
- 0 min A: 90 B: 0 C:10
- 45 min A: 50 B:40 C:10
- 45.01 min A:10 B: 80 C: 10
- 50 min A:10 B: 80 C:10

High pH Separation Conditions
- A: H_2O
- B: MeCN
- C: 100 mM NH_4OH, pH 10.0
- 0 min A: 90 B: 0 C:10
- 45 min A: 50 B:40 C:10
- 45.01 min A:10 B: 80 C: 10
- 50 min A:10 B: 80 C:10

Conclusions

pH substantially influences the retention of tryptic peptides on XTerra® MS C18 columns.

The changes in elution order make the same column chemistry orthogonal by varying pH of the mobile phase.

The reversed-phase chemistries tested at the same pH are nearly linear.

There were substantial differences in peak capacity observed between different reversed-phase columns at low pH. The Polarity™ dC18 is the best for low pH peptide separations.

100 angstrom pore size columns outperform 300 angstrom columns for most tryptic peptide separations.

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