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Development and Evaluation of a High Peak Capacity Reversed-Phase HPLC Column for the Separation of Peptides under LC/MS Conditions

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Introduction

The separation and characterization of peptides derived from protein tryptic digest by reversed–phase HPLC coupled with mass spectrometry has become an important tool in the study of the proteome. This however, requires mass spectrometry compatible methodologies in order to eliminate ion suppression and a subsequent loss of sensitivity by the mass spectrometer. We have developed a high resolution, high peak capacity column that does not require TFA for the separation of peptides. A comparison of our Atlantis™ dC18 column to other commercially available columns under LC/MS compatible conditions was evaluated. The column was found to have increased peak capacity as compared to all others tested for peptide mapping.

Experimental

Waters PDA 996

Waters Alliance™ HPLC

Waters ZQ™ Mass Detector
–Source = ESI (+), Capillary (kV) = 3.3, Cone (V) = 30
–Temperature (°C), Source = 150, Desolvation = 350
–Gas flow (L/Hr), Cone = 50, Desolvation = 500
–Scan Mode

Micromass Q-Tof II
–Source = ESI (+)
–Capillary (kV) = 3.3
–Cone (V) = 30
–Collision Energy=30

Separation Method UV/MS:
–A: 0.1% Formic acid in water
–B: 0.1% Formic acid in MeCN

or

–A: 0.020% TFA in water
–B: 0.016% TFA in MeCN
–Gradient from 0% B to 40% B in 45 min.
–0.75 ml/min, split flow to ~0.2ml/min diverted to ZQ

Reversed-Phase Columns Tested
–Atlantis™ dC18 4.6 x 50 mm 5µm
–Wide Variety of Columns "Market" as Peptide Columns 4.6 x 50 mm 5µm

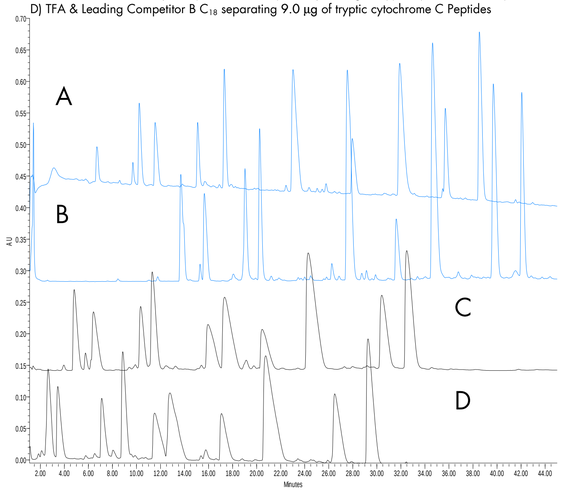
The Average USP Tailing Factor and Average Peak Capacity for a wide variety of Reversed Phased C_{18} Chemistries "Marketed" as Peptide Separation Columns by the Leading Manufaturers.

Columns tested using Formic Acid in a H_2O / ACN gradient (see methods) with a standard nine peptide mixture at 4.5µg mass load

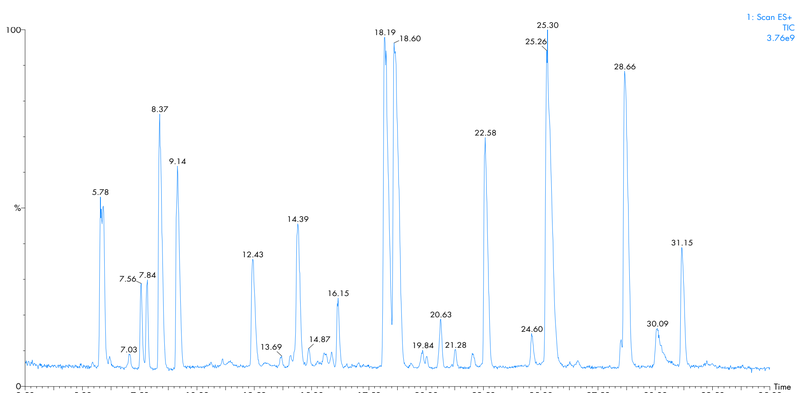
Column	Avg. USP TF	Avg. P (50%)
Atlantis™ dC18	*1.23*	*309.2*
Leading Competitor A (Chemistry1)	*1.22*	*300.2*
Leading Competitor A (Chemistry 2)	1.27	273.7
Prototype	1.42	250.0
Leading Competitor E (Chemistry 1)	1.33	250.6
Leading Competitor C (Chemistry 1)	1.26	237.9
Leading Competitor A (Chemistry 3)	1.22	210.9
Leading Competitor D (Chemistry 1)	1.47	196.2
Leading Competitor B (Chemistry 1)	1.47	204.5
Leading Competitor B (Chemistry 2)	1.53	187.8
Leading competitor E (Chemistry 2)	1.79	152.7
Leading Competitor A (Chemistry 4)	1.81	127.9
Competitor F (Chemistry 1)	1.58	126.3
Leading Competitor A (Chemistry 5)	2.24	107.4

The Effects of Formic Acid as compared with TFA on Peak Shape, Peak Capacity, and Retention Time between Atlantis™ dC18 (100Å) and the Leading Peptide C_{18} Column (nominal 100Å)

A) Formic Acid & Atlantis™ dC18 separating 9.0 µg of tryptic cytochrome C Peptides
B) TFA & Atlantis™ dC18 separating 9.0 µg of tryptic cytochrome C Peptides
C) Formic Acid & Leading Competitor B C_{18} separating 9.0 µg of tryptic cytochrome C Peptides
D) TFA & Leading Competitor B C_{18} separating 9.0 µg of tryptic cytochrome C Peptides



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



The Effects of Formic Acid compared with TFA on Peak Capacity between Reversed Phase Columns for the LC/MS analysis of peptides.

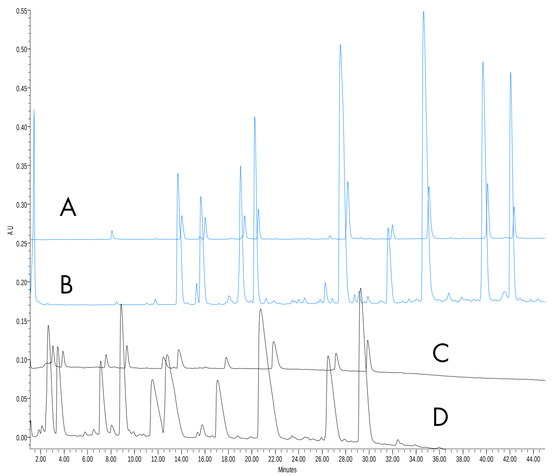
Mobile Phase A:H_2O with 0.100% Formic Acid Mobile Phase B: ACN with 0.065% Formic Acid			
Peak #	Column A (300 Å)	Column A (100Å)	Atlantis™ dC18 (100Å)
1	0.319	0.360	0.260
2	0.436	0.296	0.300
3	0.343	0.261	0.259
4	0.304	0.244	0.234
5	0.500	0.341	0.313
6	0.732	0.580	0.492
7	0.671	0.446	0.377
8	0.499	0.319	0.269
Peak Capacity*	95.9	126.4	143.8

Mobile Phase A:H_2O with 0.020% TFA Mobile Phase B: ACN with 0.016% TFA			
Peak #	Column A (300 Å)	Column A (100Å)	Polarity™ dC18 (100Å)
1	0.323	0.28	0.326
2	0.389	0.308	0.321
3	0.283	0.91	0.267
4	0.269	0.288	0.251
5	0.400	0.409	0.362
6	0.516	0.519	0.443
7	0.414	0.439	0.377
8	0.318	0.35	0.309
Peak Capacity*	123.6	124.8	135.5
%Δ in Peak Capacity Formic/TFA*	*78%*	*101%*	*106%*

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

Differences in Peak Shape, Peak Capacity, and Retention between Atlantis™ dC18 (100Å) and the Leading Peptide C18 Column (nominal100Å) run in LC/MS conditions under different mass loads.

A) Atlantis™ dC18 separating 4.5 µg of tryptic cytochrome C Peptides
B) Atlantis™ dC18 separating 45.0 µg of tryptic cytochrome C Peptides
C) Leading Competitor B C_{18} separating 4.5µg of tryptic cytochrome C Peptides
D) Leading Competitor B C_{18} separating 45.0 µg of tryptic cytochrome C Peptides



Conclusions

- 100Å reversed-phase columns outperform 300Å columns for the vast majority of peptides obtained from enzymatic digestion of proteins under LC/MS separation conditions regardless of manufacturer.
- Atlantis™ dC18 and a very few other columns retain their superior performance characteristics under LC/MS conditions using Formic Acid.
- **Atlantis™** dC18 retains it's superior performance characteristics under increasing mass loads while the majority of other columns quickly deteriorate
- Superior retention of Hydrophilic and all peptides