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Column Benchmarking for Peptide Separation by Reversed-phase High Performance Liquid Chromatography

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Introduction

Reversed-phase high performance liquid chromatography (RP-HPLC) is the primary method used for the separation of peptides. It is used for peptide mapping by UV or liquid chromatography-mass spectrometry (LC-MS), and isolation of natural and synthetic peptides. Both applications rely on efficient RP-HPLC separation. In the case of peptide mapping, the ability to separate tens and hundreds of peaks is required.

A column benchmark was performed for the RP-HPLC separation of a peptide mixture (Table 1). We chose 9 biologically active peptides which have a wide range of hydrophobicities, molecular weights, and isoelectric points. This is a representative sample which allows us to challenge a column's ability to separate peptides without using a complicated protein digest.

We evaluated column performance based on column peak capacity. Peak capacity (P) is defined as the number of peaks a column can separate within a given gradient time. An example of the calculation for peak capacity is shown in Figure 1. This calculation was used to determine the P of various Waters and competitor columns (Table 2) based on their separation of the peptide mixture.

The columns that performed well for separation of the peptide mixture were subjected to the RP-HPLC separation of a tryptic digest of bovine serum albumin (BSA) using UV and LC-MS compatible mobile phases (Figures 3 and 4, respectively). Trifluoroacetic acid (TFA) is the preferred ion pairing agent for peptide separation because it gives symmetrical peak shape, superior resolution, and greater retention. However, in our experience, TFA causes a two to four-fold reduction in MS signal intensity when it is used in the mobile phase. Instead, formic acid was used as a mobile phase additive for LC-MS applications.

We also evaluated the effect of mass load on column peak capacity. As mass load increases, peak capacity decreases (Figure 5). A column's mass load capacity in overloading conditions is important for the semi-preparative isolation of native and synthetic peptides.

Experimental

HPLC System: Alliance® 2690 equipped with a 996 photodiode array detector (Waters)

Columns: All columns were in the 4.6 x 50 mm, 5 µm, C_{18} configuration unless otherwise noted on figures and tables.

Column Temp.: 40 °C

Flow rate: 0.75 ml per minute

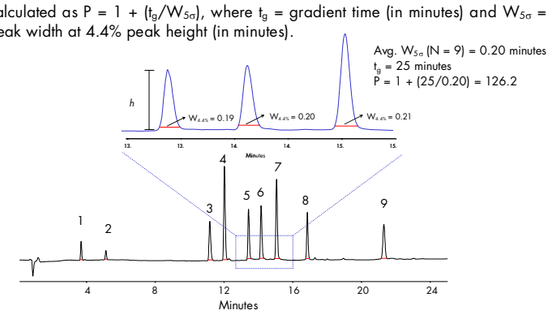
Samples: A mixture of 9 biologically active peptides was prepared. The molecular weights, isoelectric points, and hydrophobicities of these peptides can be found in Table 1. Bovine serum albumin (BSA) tryptic digest was prepared by digesting BSA (Sigma) overnight at 37°C. Trypsin:BSA ratio was 1:50 (w:w). Digestion was quenched with ~0.5% TFA (pH < 4). Storage buffer consisted of 50 mM NH_4HCO_3.

Table 1: Nine biologically active peptides used for column benchmarking study

Peptide	# of AA	MW	pI	Hydrophobicity (Meek)[a]
1. L2275	9	986.6	12.04	2.8
2. A6677	10	1001.5	9.34	3.0
3. Bradykinin	9	1060.2	12.00	32.6
4. Angiotensin II	8	1046.2	7.35	43.7
5. Angiotensin I	10	1296.5	7.51	44.4
6. Substance P	11	1347.6	11.20	63.5
7. Renin Substrate	14	1759.0	7.61	50.5
8. Insulin B chain	30	3495.9	7.51	68.5
9. Melittin	22	2845.8	12.06	130.1

[a]The theoretical hydrophobicity index (J. L. Meek and Z. L. Rossetti, *J. Liquid Chromatogr.*, 211 (1981) 15-28.) predicts peptide retention in minutes. This is purely theoretical and depends on the HPLC conditions. In this study, the prediction correlated well with the actual retention.

Figure 1: Example of column peak capacity (P) calculation. Peak capacity was calculated as $P = 1 + (t_g/W_{5\sigma})$, where t_g = gradient time (in minutes) and $W_{5\sigma}$ = peak width at 4.4% peak height (in minutes).

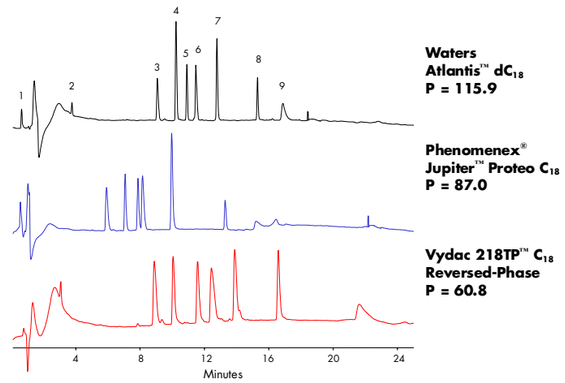


Peak capacities for various Waters and competitor columns were calculated as shown in Figure 1. The peak widths of 9 peaks were averaged in order to calculate the peak capacity values found in Table 2.

Table 2: Average column peak capacities in 0.1% TFA and 0.1% formic acid conditions (4.5 µg total mass load)

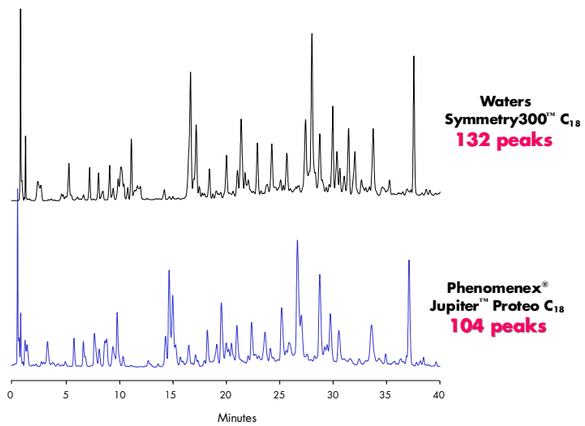
Column	Peak Capacity (0.1% TFA)	Peak Capacity (0.1% Formic Acid)
Waters Atlantis™ dC_{18} (100Å)	114.3	115.9
Waters YMC™ ODS-AQ (120Å)	-------	112.0
Waters Symmetry® C_{18} (100Å)	129.0	111.0
Waters SymmetryShield™ RP_{18} (100Å)	125.5	111.0
Vydac 238MS™ LC/MS C_{18} Reversed-Phase (300Å)	110.8	105.8
Waters Symmetry300™ C_{18} (300Å)	117.4	100.4
Vydac 218MS™ LC/MS C_{18} Reversed-Phase (300Å)	111.8	100.3
Agilent Zorbax® 300SB-C_{18} (300Å)	-------	95.1
Phenomenex® Jupiter™ C_{18} (300Å)	-------	94.9
Phenomenex® Jupiter™ Proteo C_{18} (4 µm, 90Å)	93.1	87.0
Polymer Laboratories PLRP-S (300Å)	-------	82.8
Waters XTerra® MS C_{18} (120Å)	117.6	75.2
Vydac 218TP™ C_{18} Reversed-Phase (300Å)	114.1	60.8
Vydac 238TP™ C_{18} Reversed-Phase (300Å)	114.7	52.1

Figure 2: Peptide separation in 0.1% formic acid (4.5 µg total mass load)



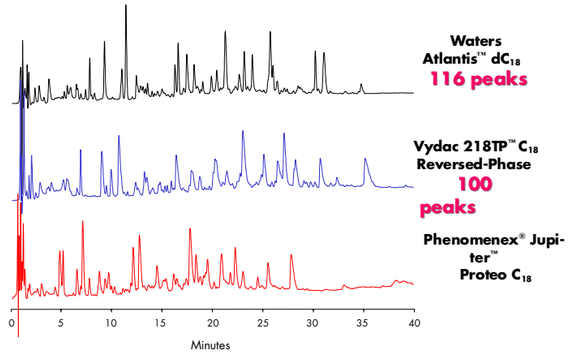
Mobile phase A: 0.1% formic acid in water, B: 0.065% formic acid in acetonitrile. Gradient from 0-50% B in 25 minutes, 0.75 ml/min., 40°C, UV 220 nm.

Figure 3: BSA tryptic digest separation in 0.1% TFA (50µg total mass load)



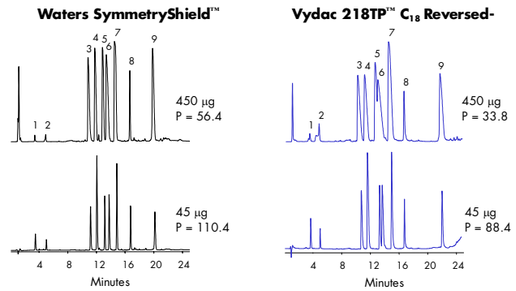
Mobile phase A: 0.1% TFA in water, B: 0.08% TFA in acetonitrile. Gradient from 0-40% B in 45 minutes, 0.75 ml/min., 40°C, UV 220 nm.

Figure 4: BSA tryptic digest separation in 0.1% formic acid (50µg total mass



Mobile phase A: 0.1% formic acid in water, B: 0.065% formic acid in acetonitrile. Gradient from 0-40% B in 45 minutes, 0.75 ml/min., 40°C, UV 220 nm.

Figure 5: Column peak capacity increases with decreasing mass load (mass load shown is total mass load injected for all 9 peptides)



Mobile phase A: 0.1% TFA in water, B: 0.08% TFA in acetonitrile. Gradient from 0-50% B in 25 minutes, 0.75 ml/min., 40°C, UV 220 nm.

Conclusions

- Atlantis™ dC_{18} and YMC™ ODS-AQ display excellent performance in formic acid conditions, and are the best choice of columns for LC-MS applications.
- SymmetryShield™ RP_{18} preserves peak shape at increased mass loads, and is recommended for semi-preparative peptide purifications.
- High column peak capacity is important for peptide mapping applications. Atlantis™ dC_{18}, YMC™ ODS-AQ, and Symmetry® C_{18} columns offer excellent performance for UV and LC-MS based peptide mapping.