Flow Rate, Concentration and Saturation: Investigating the Fundamentals of Sample-Trapped Column Injection

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Introduction

Sample trapped column injection is an injection strategy commonly employed in nanobore LC/MS based analysis of complex peptide mixtures. The approach of sample trapped column injection provides several inherent advantages. By effectively desalting and concentrating samples on-line, sample traps improve analytical column longevity and throughput. Additionally, the ability to load samples onto a trap column at a much higher flow rate than is feasible for a typical 75µm ID nanobore analytical column is a particularly attractive feature of this approach. Duty cycle can be significantly improved when the sample loading flow rate is decoupled from the gradient flow rate. Here we investigate the role sample trap column injection has on analyte retention and overall chromatographic performance. Using a direct flow nano-LC pump with the ability to deliver flow rates ranging from 50 nl/min to 20 µ/min coupled to an autosampler and commercially available peptide standards and protein digests, we evaluated the relationship between flow rate, analyte concentration and analyte composition to determine the effect on chromatographic performance.

Methods & Materials

Instrumentation

- Leap Technologies HTC Pal Autosampler
  - VICI 6-port micro valve
  - 1.0 µl sample loop
- VICI 10-port valve with 2 position actuator
- Thermo LCQ Deca ion trap mass spectrometer
  - MS/MS scan
  - Full MS Scan
    - 3 Microscans/spectra
    - 390.00 - 1500.00 Da mass range
- Customized Digital PicoView nanospray source
- Eksigent nanoLC-2D pump
  - Channel 1
    - Mobile Phase A = 98% water with 0.1% formic acid and 2% acetonitrile with 0.1% formic acid
    - Mobile Phase B = 98% water with 0.1% formic acid and 2% acetonitrile with 0.1% formic acid
  - Channel 2
    - Mobile Phase A = 0.1% formic acid in water
    - Mobile Phase B = 0.1% formic acid in acetonitrile

Columns

Analytical Column

- PicoFrit column (75 µm ID x 15 µm tip) packed to 10 cm with Proteopep II C18 5 µm resin

Trap Column

- IntegraFrit trap column (100 µm ID) packed to 2.5 cm with Proteopep II C18 5 µm resin

Samples

- Equimolar mix of four peptides, variable concentrations as indicated
  - Angiotensin I, 1296 Da
  - Angiotensin II, 1045 Da
  - Val^5-Angiotensin I, 1282 Da
  - Neurotensin, 1672 Da
- BSA digest (Waters MassPrep)
  - 300 fmol/µL in 0.1% Formic acid

Conclusions

- The maximum loading capacity of a 100 µm ID x 2.5 cm bed trap column was determined to be 130 ng using a mixture of 4 standard peptides
  - 130 ng Polypeptide represents column saturation for these column dimensions
  - Trap column saturation negatively impacts downstream analytical column performance
- Cycle time improvements demonstrated using trap column loading flow rates up to 10 µL/min.
  - Peptide recovery validated at flow rates ranging from 1–10 µL/min.
  - Adjustment of injection time for flow rate is required for maximum peptide recovery
- Increasing the amount of solvent flowing through the column at higher flow rates can lead to loss of early eluting peptides

Name	Sequence	MW (Da)	RT (min)
Angiotensin II	DRVYIHPF	1046.18	16.7
Angiotensin I	DRVYIHPFHL	1296.50	17.2
Neurotensin	pQLYQNKPRRPYIL	1672.92	17.7
Val5-Angiotensin I	DRVYVHPFHL	1282.45	18.2

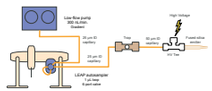
4 Peptide standard information – peptide sequence for each peptide.

Future Work

- Evaluate the loading capacity of trap columns with targeted MS/MS scans
- Investigate trap column performance at flow rates higher than 10 µl/min
- Incorporate studies for trap columns with other dimensions – for example 75 and 150 µm inner diameter
- Evaluate the performance of trap column for sample concentration
- Study retention of early eluting hydrophilic peptides on different types of resin

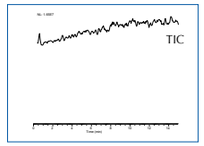
Flow Rate Evaluation

WHAT IS THE OPTIMAL FLOW RATE FOR SAMPLE TRAP LOADING?

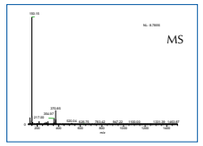


Flow Rate (µL/min)	Injection Time (min)	Total Volume (µL)	Peptides Detected
1.0	5:00	5	No
2.0	5:00	10	No
5.0	5:00	25	No
10.0	5:00	50	Yes
1.0	10:00	10	No
2.0	5:00	10	No
5.0	2:00	10	No
10.0	1:00	10	Yes

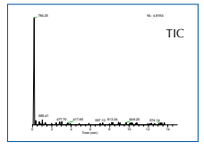
Data collected for 200 fmol/µL 4 peptide mixture at different flow rates. No peptides are detected up to 5 µL/min. flow rates. Some peptides are detected at 10 µL/min. in targeted MS/MS scan but not in full MS scan

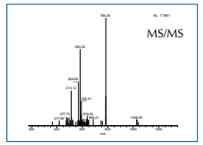


TIC chromatogram for 200 fmol/µL 4 peptide injection at 10 µL/min.



Full MS spectra for 200 fmol/µL 4 peptide injection at 10 µL/min. No peaks detected.



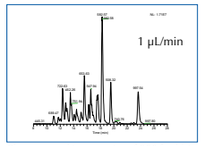


MS/MS data for 200 fmol/µL 4 peptide injection at 10 µL/min. Angiotensin I is detected at the beginning of the chromatogram.

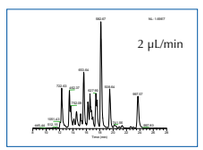
HOW DOES FLOW RATE AFFECT PEAK SHAPE?

Flow Rate (µL/min)	Injection Time (min)	m/z (Da)	Apex RT (min)	Start RT (min)	End RT (min)	Peak Area	Peak Height	Asymmetry	Peak Width (s)
1.0	5:00	722.8	12.31	12.18	12.81	9.63E+07	5.72E+06	3.85	37.80
		653.9	15.60	15.43	15.99	1.15E+08	7.72E+06	2.29	33.60
		582.9	18.25	18.09	18.57	2.52E+08	1.71E+07	2.00	28.80
		997.8	23.64	23.42	23.98	8.59E+07	5.16E+06	1.55	33.60
2.0	5:00	722.8	12.32	12.15	12.65	8.78E+07	6.33E+06	1.94	30.00
		653.9	15.61	15.44	15.92	1.31E+08	9.71E+06	1.82	28.80
		582.9	18.17	18.01	18.49	2.45E+08	1.68E+07	2.00	28.80
		997.8	23.70	23.47	24.06	8.76E+07	5.06E+06	1.57	35.40
5.0	5:00	722.8	12.43	12.30	12.65	6.87E+07	6.64E+06	1.69	21.00
		653.9	15.74	15.58	16.09	1.08E+08	7.78E+06	2.19	30.60
		582.9	18.28	18.12	18.60	2.17E+08	1.50E+07	2.00	28.80
		997.8	23.72	23.49	24.10	7.67E+07	4.44E+06	1.65	36.60
10.0	5:00	722.8	Peak not detected						
		653.9	15.93	15.77	16.30	1.12E+08	7.75E+06	2.31	31.80
		582.9	18.51	18.34	18.77	1.95E+08	1.46E+07	1.53	25.80
		997.8	23.89	23.66	24.22	6.93E+07	4.19E+06	1.43	33.60

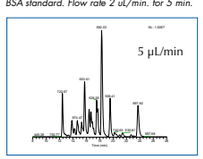
Peptide data extracted from 300 fmol/µL BSA standard chromatograms. Peptides were chosen from across the chromatogram. The loading time is constant; the flow rates are different.



Chromatographic separation of 300 fmol/µL BSA standard. Flow rate 1 uL/min. for 5 min.



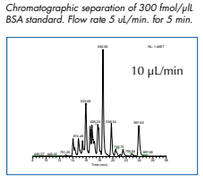
Chromatographic separation of 300 fmol/µL BSA standard. Flow rate 2 uL/min. for 5 min.



Chromatographic separation of 300 fmol/µL BSA standard. Flow rate 5 uL/min. for 5 min.

Flow Rate (µL/min)	Injection Time (min)	m/z (Da)	Apex RT (min)	Start RT (min)	End RT (min)	Peak Area	Peak Height	Asymmetry	Peak Width (s)
1.0	10:00	722.8	12.63	12.46	12.98	7.37E+07	5.43E+06	2.12	31.56
		653.9	15.88	15.70	16.14	8.59E+07	6.37E+06	1.46	26.76
		582.9	18.47	18.30	18.74	1.66E+08	1.20E07	1.51	26.40
		997.8	23.93	23.66	24.28	2.66E+07	1.44E+06	1.26	37.08
2.0	5:00	722.8	12.46	12.27	12.89	7.30E+07	4.58E+06	2.22	37.20
		653.9	15.76	15.57	16.03	7.83E+07	5.88E+06	1.37	27.84
		582.9	18.35	18.17	18.61	1.50E+08	1.11E+07	1.49	26.52
		997.8	23.81	23.55	24.21	4.06E+07	2.12E+06	1.63	39.36
5.0	2:00	722.8	12.63	12.45	13.08	8.69E+07	5.67E+06	2.46	37.68
		653.9	15.85	15.68	16.16	1.09E+08	8.10E+06	1.77	28.44
		582.9	18.46	18.28	18.74	1.76E+08	1.21E+07	1.56	27.84
		997.8	23.89	23.63	24.28	3.57E+07	1.83E+06	1.50	39.00
10.0	1:00	722.8	12.71	12.53	13.11	9.95E+07	7.07E+06	2.23	34.80
		653.9	15.82	15.64	16.13	1.05E+08	7.57E+06	1.70	29.52
		582.9	18.43	18.26	18.71	1.72E+08	1.22E+07	1.74	27.24
		997.8	23.87	23.62	24.27	5.56E+07	2.87E+06	1.62	39.24

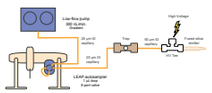
Peptide data extracted from 300 fmol/µL BSA standard chromatograms. Peptides were chosen from across the chromatogram. The total amount of solvent flowing through the trap column is kept at 10 µL, loading time and loading flow rate vary.



Chromatographic separation of 300 fmol/µL BSA standard. Flow rate 10 uL/min. for 5 min.

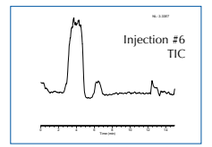
Trap Column Capacity Evaluation

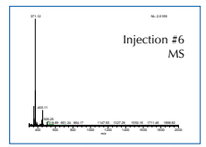
HOW MUCH SAMPLE CAN BE LOADED ONTO THE TRAP COLUMN?



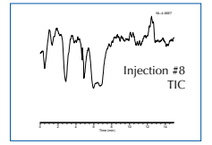
Injection Number	Peptide Concentration (pmol/µL)	Total Mass (ng)	Flow Rate (nL/min.)	Peptides Detected
1	5	26.5	500	No
2	5	53.0	500	No
3	5	79.5	500	No
4	5	132.5	500	No
5	5	159.0	500	No
6	5	185.5	500	No
7	5	212.0	500	Yes
1	20	106.0	500	No
1	25	131.3	500	No
1	30	157.6	500	Yes
1	35	183.9	500	Yes

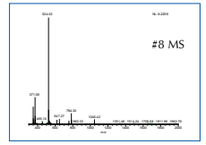
Data was collected for replicate injections of a 4-peptide mixture at different concentrations. 5 pmol/µL of 4-Peptide mixture was injected onto the column until peptide peaks were detected. Single injection of higher concentration 4 peptide mixture was injected onto the trap column. Peptide peaks were detected in 30pmol/µL and 35 pmol/µL injections.





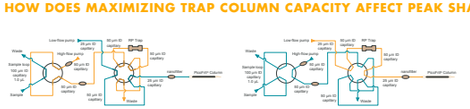
TIC chromatogram and MS spectra for 4 peptide standard. No peptides were detected in this injection.





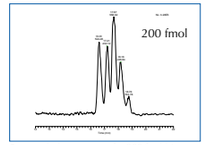
TIC chromatogram and MS spectra for 4 peptide standard. Peptide ions are detected in this injection. Column is overloaded and peptides are coming from the column without retention.

HOW DOES MAXIMIZING TRAP COLUMN CAPACITY AFFECT PEAK SHAPE?

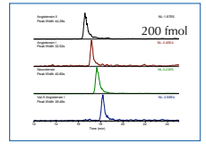


Flow Rate (µL/min)	Loading Time (min)	Peptide Name	m/z (Da)	Apex RT (min)	Start RT (min)	End RT (min)	Peak Area	Peak Height	Asym.	Peak Width (s)
200 fmol/µL Peptide Mixture										
1.0	10:00	Angiotensin II	524	16.53	16.32	17.09	5.77E+06	2.78E+05	46.20	39.0
		Angiotensin I	433	17.18	16.99	17.54	4.60E+05	2.83E+04	32.85	54.8
		Neurotensin	558	17.52	17.31	17.97	3.32E+06	1.63E+05	39.60	45.5
		Val5-Angiotensin I	428	18.16	17.94	18.68	4.28E+05	2.05E+04	44.70	40.3
2.0	5:00	Angiotensin II	524	16.64	16.42	17.16	5.73E+06	2.80E+05	44.28	40.7
		Angiotensin I	433	17.18	16.99	17.53	4.64E+05	2.73E+04	32.52	55.4
		Neurotensin	558	17.61	17.40	18.08	1.50E+07	7.14E+05	40.80	44.1
		Val5-Angiotensin I	428	18.17	17.94	18.59	5.21E+05	2.79E+04	39.48	45.6
5.0	2:00	Angiotensin II	524	16.85	16.59	17.41	6.87E+06	2.92E+05	48.84	36.9
		Angiotensin I	433	17.37	17.18	17.79	5.45E+05	3.14E+04	36.48	49.3
		Neurotensin	558	17.78	17.58	18.29	3.92E+07	1.89E+06	42.72	42.1
		Val5-Angiotensin I	428	18.37	18.15	18.75	5.63E+05	2.90E+04	36.36	49.5
10.0	1:00	Angiotensin II	524	16.53	16.29	17.07	6.65E+06	2.92E+05	47.28	38.1
		Angiotensin I	433	17.07	16.88	17.38	5.17E+05	3.41E+04	30.36	59.3
		Neurotensin	558	17.49	17.25	17.93	4.06E+07	2.03E+06	40.80	44.1
		Val5-Angiotensin I	428	18.03	17.78	18.44	5.79E+05	2.92E+04	39.72	45.3
20 pmol/µL 4 Peptide Mixture										
1.0	10:00	Angiotensin II	524	15.25	15.16	15.56	3.24E+09	2.03E+08	24.00	75.0
		Angiotensin I	433	15.75	15.46	16.31	4.15E+09	1.21E+08	51.00	35.3
		Neurotensin	558	16.49	16.27	17.99	4.00E+09	8.78E+07	103.20	17.4
		Val5-Angiotensin I	428	16.62	16.53	17.42	1.84E+09	5.77E+07	53.40	33.7
2.0	5:00	Angiotensin II	524	15.08	14.88	15.25	3.26E+09	2.00E+08	22.20	81.1
		Angiotensin I	433	15.30	15.16	16.03	4.67E+09	1.16E+08	52.20	34.5
		Neurotensin	558	16.16	15.99	17.72	3.70E+09	7.63E+07	103.80	17.3
		Val5-Angiotensin I	428	16.40	16.25	17.62	2.80E+09	6.24E+07	82.20	21.9
5.0	2:00	Angiotensin II	524	15.12	14.88	15.28	3.63E+09	2.01E+08	24.00	75.0
		Angiotensin I	433	15.48	15.17	16.08	4.67E+09	1.14E+08	54.60	33.0
		Neurotensin	558	16.14	16.02	17.20	2.87E+09	7.73E+07	70.80	25.4
		Val5-Angiotensin I	428	16.39	16.30	17.19	2.15E+09	5.90E+07	53.40	33.7
10.0	1:00	Angiotensin II	524	15.14	15.07	15.46	3.22E+09	2.04E+08	23.40	76.9
		Angiotensin I	433	15.47	15.35	16.23	4.52E+09	1.12E+08	52.80	34.1
		Neurotensin	558	16.40	16.17	17.89	3.78E+09	8.62E+07	103.20	17.4
		Val5-Angiotensin I	428	16.55	16.46	17.35	2.09E+09	5.57E+07	53.40	33.7

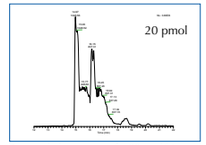
Peak capacity data calculated for 200 fmol/µL and 20pmol/µL 4 peptide standards. Chromatograms were collected at different loading times and flow rates. Total amount of solvent flowing through the trap column is 10 µL.



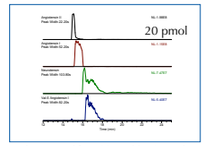
Base peak chromatogram for 200 fmol/µL 4-peptide standard. Loading: 5 min. at 2 µL/min.



Peaks extracted from 200 fmol/µL 4-peptide chromatogram. Loading: 5 min. at 2 µL/min.



Base peak chromatogram for 20 pmol/µL 4-peptide standard. Loading: 5 min. at 2 µL/min.



Peaks extracted from 20 pmol/µL 4-peptide chromatogram. Loading: 5 min. at 2 µL/min.