Nanobore RPHPLC: Determining The Role of Selectivity in Method Development

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Introduction

Nanobore chromatography coupled with ESI mass spectrometry has proven to be an excellent approach for both protein identification and protein quantitation in the fields of proteomics and biomarker discovery. The growth of quantitation and quantitative approaches employing nanobore LC-MS stimulates the need for robust separations with excellent RSDs on the nanobore scale. In recent years, the introduction of sub-2 µm stationary phases and UHPLC has enabled scientists to perform highly efficient separations, necessary for the analysis of highly complex biological matrices. Regardless of particle size, predicting the selectivity of a specific stationary phase for a specific analyte is virtually impossible. Selecting the correct stationary phase for analytical separation is a key part of method development and method optimization. Here we survey a sample of the many different stationary phases available in nanobore column format to evaluate their effect on selectivity. All experimental variables were held constant while the stationary phase—or the separation it generated under these conditions—was the variable. 100 fmol of a commercially available BSA digest was injected onto a 10 cm, 75 µm ID column and separated by an acetonitrile gradient of 2 – 50% B at 300 nL/min. over 30 minutes. Resins comprised of 5 µm particles were employed to maintain robustness and flexibility. Multiple chemistries on the same solid support were gauged. Separations were evaluated based on their retention factor and separation factor, as well as peak shape and the RSD of these values over a series of 10 injections.

Materials & Methods

Chromatography

- Leap Technologies HTC Pal autosampler
  - ~ VICI 6-port micro valve
  - ~ 1.0 µL sample loop
- Eksigent nanoLC-2D pump Channel 2
  - ~ Mobile phase A = 0.1% formic acid in water
  - ~ Mobile phase B = 0.1% formic acid in acetonitrile
    - · On-column injection
    - · Load at 1000 nL/min. for 5 minutes
    - · 300 nL/min. Gradient elution
    - · 30-Minute gradient: 2% to 50% B

Mass Spectrometry

- Thermo LCQ Deca ion trap mass spectrometer
- 3 Microscans/spectra
- 390.00 – 1500.00 Da mass range for MS spectra
- New Objective DPV-150 Digital PicoView nanospray source

Analytical Column

- 75 µm ID PicoFrit column with a 15 µm integral frit; slurry packed to 10 cm with selected chemistries

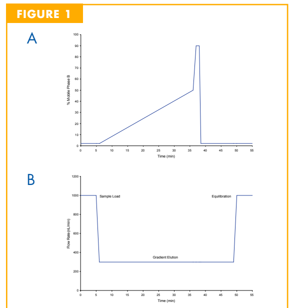
Samples

- Enolase digest (Waters MassPrep)
  - ~ 300 fmol/µL in 0.1% formic acid
- Equimolar mix of four peptides
  - ~ 200 fmol/µL in 0.1% formic acid/H_2O
    - · Angiotensin I, 1296 Da
    - · Angiotensin II, 1045 Da
    - · Val[5]-Angiotensin I, 1282 Da
    - · Neurotensin, 1672 Da

Media Code	Actual Bed Length (mm)	Chemistry: Brand Name	Chemistry: Particle Size (µm)	Chemistry: Pore Size (Å)	Chemistry: Chemistry	Pressure: Flow Rate (nL/min.)	Pressure: Mobile Phase	Pressure: Pc (PSI)
H001	100	ProteoPep	5	300	C18	1,000	2% B	1,108
H002	98	ProteoPep II	5	300	C18			1,166
H035	100	HALO	2.7	90	C18			3,122
H039	98	HALO Peptide	2.7	160	ES-C18			3,517
H052	102	BioBasic	5	300	C18			2,415
H062	99	Hypercarb	5	250	PGC			1,639
H070	100	Hypersil GOLD	5	175	C18			1,338
H072	102	Hypersil GOLD	5	175	PFP			2,449
H074	103	Hypersil GOLD	5	175	C18AQ			1,302

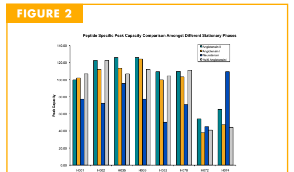
Table 1 PicoFrit stationary phase details and column pressure values. PicoFrit column specification: 75 µm ID, 15 µm tip with a nominal bed length of 100 mm

FIGURE 1



Gradient and flow rate profiles used for data collection. A) 55 min gradient profile B) flow rate profile

FIGURE 2

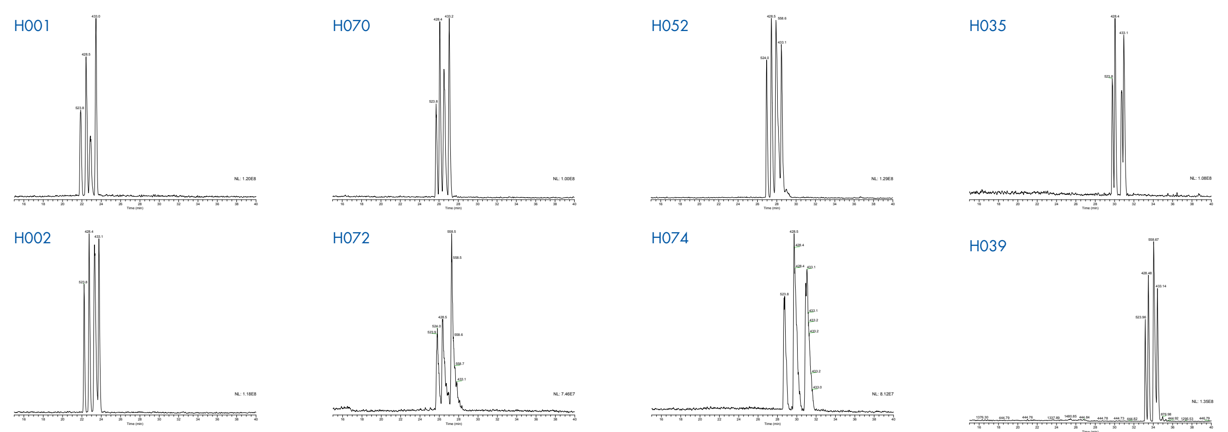


Plot of peptide specific peak capacity calculated for four standard peptides separated on a 75um ID, 10 cm PicoFrit column packed with eight different reversed phase materials

Stationary Phase	Peptide Name	m/z (Da)	Peptide Sequence	Apex RT (min)	Start RT (min)	End RT (min)	Area	Height	Peak Width (min)	Peak Capacity
H001	Angiotensin II	524	DRVYIHPF	21.94	21.80	22.11	7.18E+08	7.06E+07	0.30	99.90
	Angiotensin I	433	DRVYIHPFHL	22.54	22.41	22.70	5.36E+08	5.66E+07	0.30	102.12
	Neurotensin	558	pELYENKPRRPYIL	22.96	22.81	23.21	5.38E+08	4.02E+07	0.39	77.27
	Val5-Angiotensin I	428	DRVYVHPFHL	23.52	23.40	23.69	5.60E+08	5.84E+07	0.28	106.88
H002	Angiotensin II	524	DRVYIHPF	22.28	22.18	22.42	5.76E+08	7.31E+07	0.25	122.62
	Angiotensin I	433	DRVYIHPFHL	22.80	22.69	22.96	2.72E+08	3.20E+07	0.27	112.11
	Neurotensin	558	pELYENKPRRPYIL	23.32	23.20	23.62	1.24E+09	8.79E+07	0.42	72.43
	Val5-Angiotensin I	428	DRVYVHPFHL	23.79	23.70	23.94	2.89E+08	3.60E+07	0.25	122.62
H035	Angiotensin II	524	DRVYIHPF	29.71	29.63	29.87	5.63E+08	6.78E+07	0.24	126.00
	Angiotensin I	433	DRVYIHPFHL	30.00	29.91	30.17	2.56E+08	2.84E+07	0.27	113.50
	Neurotensin	558	pELYENKPRRPYIL	30.68	30.58	30.90	6.21E+08	5.89E+07	0.32	95.74
	Val5-Angiotensin I	428	DRVYVHPFHL	30.91	30.83	31.11	2.63E+08	2.81E+07	0.28	106.88
H039	Angiotensin II	524	DRVYIHPF	33.09	33.02	33.26	5.43E+08	6.97E+07	0.24	126.00
	Angiotensin I	433	DRVYIHPFHL	33.41	33.32	33.56	2.38E+08	3.02E+07	0.24	124.29
	Neurotensin	558	pELYENKPRRPYIL	33.97	33.87	34.26	1.57E+09	1.26E+08	0.39	77.27
	Val5-Angiotensin I	428	DRVYVHPFHL	34.36	34.28	34.55	2.47E+08	2.91E+07	0.27	112.11
H052	Angiotensin II	524	DRVYIHPF	27.10	27.01	27.28	8.42E+08	9.52E+07	0.28	109.43
	Angiotensin I	433	DRVYIHPFHL	27.61	27.50	27.81	4.10E+08	4.34E+07	0.30	99.90
	Neurotensin	558	pELYENKPRRPYIL	28.10	27.98	28.59	2.11E+09	1.08E+08	0.61	50.18
	Val5-Angiotensin I	428	DRVYVHPFHL	28.63	28.55	28.84	2.81E+08	3.11E+07	0.29	104.45
H070	Angiotensin II	524	DRVYIHPF	25.65	25.55	25.82	5.84E+08	6.37E+07	0.27	109.76
	Angiotensin I	433	DRVYIHPFHL	25.99	25.90	26.19	2.61E+08	2.64E+07	0.29	103.45
	Neurotensin	558	pELYENKPRRPYIL	26.43	26.29	26.71	9.61E+08	7.04E+07	0.42	70.87
	Val5-Angiotensin I	428	DRVYVHPFHL	26.96	26.86	27.13	2.79E+08	3.14E+07	0.27	111.11
H072	Angiotensin II	524	DRVYIHPF	25.86	25.67	26.23	5.94E+08	3.53E+07	0.56	54.25
	Angiotensin I	433	DRVYIHPFHL	26.41	26.28	27.09	3.14E+08	1.55E+07	0.81	38.04
	Neurotensin	558	pELYENKPRRPYIL	27.33	27.21	27.89	1.10E+09	6.47E+07	0.68	45.12
	Val5-Angiotensin I	428	DRVYVHPFHL	27.44	27.26	28.01	2.82E+08	1.38E+07	0.75	41.00
H074	Angiotensin II	524	DRVYIHPF	28.72	28.61	29.08	8.38E+08	5.32E+07	0.47	65.29
	Angiotensin I	433	DRVYIHPFHL	29.78	29.63	30.28	3.14E+08	1.69E+07	0.65	47.39
	Neurotensin	558	pELYENKPRRPYIL	30.92	30.78	31.06	5.10E+08	5.67E+07	0.28	109.43
	Val5-Angiotensin I	428	DRVYVHPFHL	31.04	30.87	31.57	3.25E+08	1.48E+07	0.69	44.27

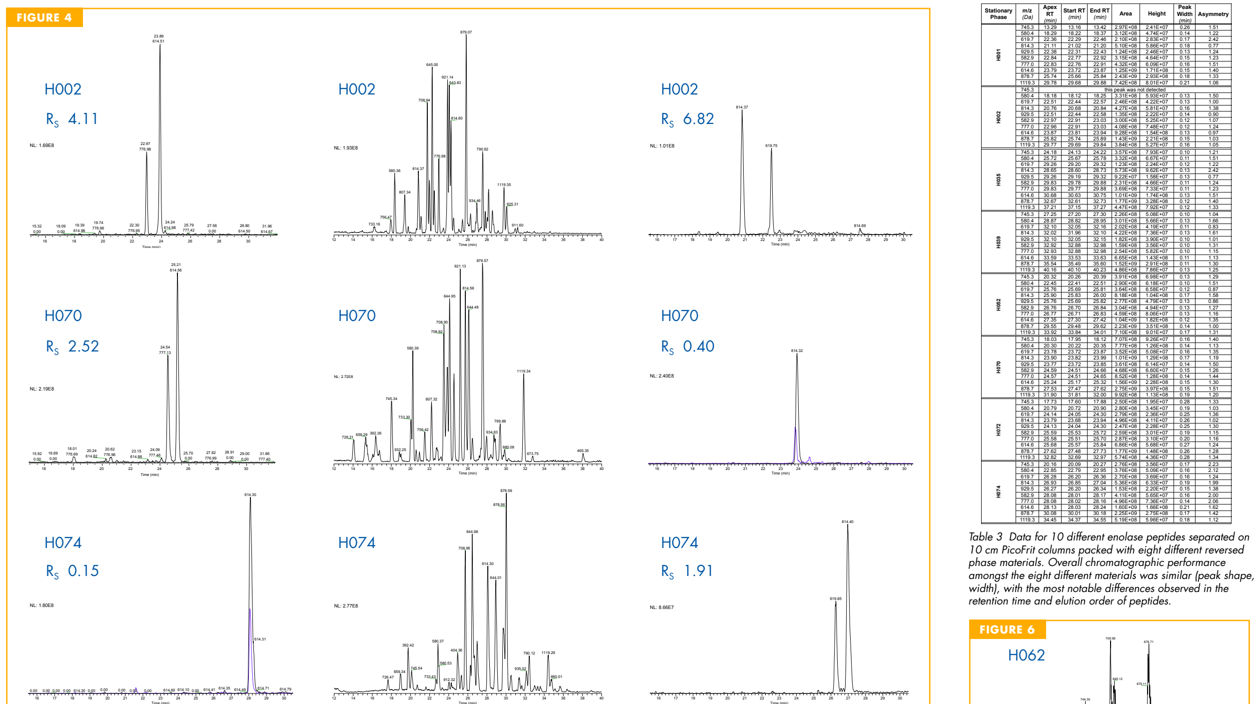
Table 2 Peptide specific peak capacity data calculated for four standard peptides separated on a 75um ID, 10 cm PicoFrit column packed with eight different reversed phase materials

FIGURE 3



Separation of four peptide mixture on eight different reversed phase materials. Differences in peak shape, intensity, elution order and retention time of the four peptides is apparent amongst the different chemistries.

FIGURE 4



Resolution data for 300 fmol on-column injection of enolase digest A) peaks with molecular weight 814.3 and 619.7 and B) peaks with molecular weight 777 and 614

Stationary Phase	m/z (Da)	Apex RT (min)	Start RT (min)	End RT (min)	Area	Height	Peak Width (min)	Asymmetry
H001	745.3	13.29	13.16	13.42	2.97E+08	2.41E+07	0.26	1.51
	580.4	18.29	18.22	18.37	3.12E+08	4.74E+07	0.14	1.22
	619.7	22.36	22.29	22.46	2.10E+08	2.83E+07	0.17	2.42
	814.3	21.11	21.02	21.20	5.10E+08	5.86E+07	0.18	0.77
	929.5	22.38	22.31	22.43	1.24E+08	2.46E+07	0.13	1.24
	582.9	22.84	22.77	22.92	3.15E+08	4.64E+07	0.15	1.23
	777.0	22.83	22.76	22.91	4.32E+08	6.09E+07	0.16	1.51
	614.6	23.79	23.72	23.87	1.25E+09	1.71E+08	0.15	1.40
	878.7	25.74	25.66	25.84	2.43E+09	2.93E+08	0.18	1.33
	1119.3	29.78	29.68	29.88	7.42E+08	8.01E+07	0.21	1.06
H002	745.3	this peak was not detected						
	580.4	18.18	18.12	18.25	3.31E+08	5.93E+07	0.13	1.50
	619.7	22.51	22.44	22.57	2.46E+08	4.22E+07	0.13	1.00
	814.3	20.76	20.68	20.84	4.27E+08	5.81E+07	0.16	1.38
	929.5	22.51	22.44	22.58	1.35E+08	2.22E+07	0.14	0.90
	582.9	22.97	22.91	23.03	3.00E+08	5.25E+07	0.12	1.07
	777.0	22.96	22.91	23.03	4.08E+08	7.48E+07	0.12	1.24
	614.6	23.87	23.81	23.94	9.28E+08	1.54E+08	0.13	0.97
	878.7	25.82	25.74	25.89	1.43E+09	2.21E+08	0.15	1.03
	1119.3	29.77	29.69	29.84	3.84E+08	5.27E+07	0.16	1.05
H035	745.3	24.18	24.13	24.22	3.57E+08	7.93E+07	0.10	1.21
	580.4	25.72	25.67	25.78	3.32E+08	6.67E+07	0.11	1.51
	619.7	29.26	29.20	29.32	1.23E+08	2.24E+07	0.12	1.22
	814.3	28.65	28.60	28.73	5.73E+08	9.62E+07	0.13	2.42
	929.5	29.26	29.19	29.32	9.22E+07	1.58E+07	0.13	0.77
	582.9	29.83	29.78	29.88	2.31E+08	4.66E+07	0.11	1.24
	777.0	29.83	29.77	29.88	3.69E+08	7.33E+07	0.11	1.23
	614.6	30.68	30.63	30.75	1.01E+09	1.74E+08	0.13	1.51
	878.7	32.67	32.61	32.73	1.77E+09	3.28E+08	0.12	1.40
	1119.3	37.21	37.15	37.27	4.47E+08	7.92E+07	0.12	1.33
H039	745.3	27.25	27.20	27.30	2.26E+08	5.08E+07	0.10	1.04
	580.4	28.87	28.82	28.95	3.01E+08	5.66E+07	0.13	1.66
	619.7	32.10	32.05	32.16	2.02E+08	4.19E+07	0.11	0.83
	814.3	32.02	31.96	32.10	4.22E+08	7.36E+07	0.13	1.61
	929.5	32.10	32.05	32.15	1.82E+08	3.90E+07	0.10	1.01
	582.9	32.92	32.88	32.98	1.59E+08	3.56E+07	0.10	1.31
	777.0	32.93	32.88	32.98	2.54E+08	5.82E+07	0.10	1.15
	614.6	33.59	33.53	33.63	6.65E+08	1.43E+08	0.11	1.13
	878.7	35.54	35.49	35.60	1.52E+09	2.91E+08	0.11	1.30
	1119.3	40.16	40.10	40.23	4.86E+08	7.86E+07	0.13	1.25
H052	745.3	20.32	20.26	20.39	3.91E+08	6.98E+07	0.13	1.29
	580.4	22.45	22.41	22.51	2.90E+08	6.18E+07	0.10	1.51
	619.7	25.76	25.69	25.81	3.64E+08	6.58E+07	0.12	0.87
	814.3	25.90	25.83	26.00	8.18E+08	1.04E+08	0.17	1.58
	929.5	25.76	25.69	25.82	2.77E+08	4.79E+07	0.13	0.86
	582.9	26.76	26.70	26.84	3.04E+08	4.94E+07	0.13	1.27
	777.0	26.77	26.71	26.83	4.59E+08	8.06E+07	0.13	1.16
	614.6	27.35	27.30	27.42	1.04E+09	1.82E+08	0.12	1.35
	878.7	29.55	29.48	29.62	2.23E+09	3.51E+08	0.14	1.00
	1119.3	33.92	33.84	34.01	7.10E+08	9.01E+07	0.17	1.31
H070	745.3	18.03	17.95	18.12	7.07E+08	9.26E+07	0.16	1.40
	580.4	20.30	20.22	20.35	7.77E+08	1.26E+08	0.14	1.13
	619.7	23.78	23.72	23.87	3.52E+08	5.08E+07	0.16	1.35
	814.3	23.90	23.82	23.99	1.01E+09	1.29E+08	0.17	1.19
	929.5	23.77	23.72	23.85	3.61E+08	6.14E+07	0.14	1.50
	582.9	24.59	24.51	24.66	4.68E+08	6.60E+07	0.15	1.26
	777.0	24.57	24.51	24.65	8.52E+08	1.28E+08	0.14	1.44
	614.6	25.24	25.17	25.32	1.56E+09	2.28E+08	0.15	1.30
	878.7	27.53	27.47	27.62	2.75E+09	3.97E+08	0.15	1.51
	1119.3	31.90	31.81	32.00	9.92E+08	1.13E+08	0.19	1.20
H072	745.3	17.73	17.60	17.88	2.50E+08	1.95E+07	0.28	1.33
	580.4	20.79	20.72	20.90	2.80E+08	3.45E+07	0.19	1.03
	619.7	24.14	24.05	24.30	2.79E+08	2.36E+07	0.25	1.36
	814.3	23.79	23.68	23.94	4.96E+08	4.11E+07	0.26	1.02
	929.5	24.13	24.04	24.30	2.47E+08	2.28E+07	0.25	1.30
	582.9	25.59	25.53	25.72	2.59E+08	3.01E+07	0.19	1.15
	777.0	25.58	25.51	25.70	2.87E+08	3.10E+07	0.20	1.16
	614.6	25.68	25.57	25.84	6.86E+08	5.68E+07	0.27	1.24
	878.7	27.62	27.48	27.73	1.77E+09	1.48E+08	0.26	1.28
	1119.3	32.82	32.69	32.97	5.74E+08	4.36E+07	0.28	1.34
H074	745.3	20.16	20.09	20.27	2.76E+08	3.56E+07	0.17	2.23
	580.4	22.85	22.79	22.95	3.76E+08	5.09E+07	0.16	2.12
	619.7	26.28	26.20	26.36	2.70E+08	3.69E+07	0.16	1.24
	814.3	26.93	26.85	27.04	5.36E+08	6.33E+07	0.19	1.99
	929.5	26.27	26.20	26.34	1.53E+08	2.20E+07	0.15	1.38
	582.9	28.08	28.01	28.17	4.11E+08	5.65E+07	0.16	2.00
	777.0	28.08	28.02	28.16	4.96E+08	7.36E+07	0.14	2.06
	614.6	28.13	28.03	28.24	1.60E+09	1.66E+08	0.21	1.62
	878.7	30.08	30.01	30.18	2.25E+09	2.75E+08	0.17	1.42
	1119.3	34.45	34.37	34.55	5.19E+08	5.96E+07	0.18	1.12

Table 3 Data for 10 different enolase peptides separated on 10 cm PicoFrit columns packed with eight different reversed phase materials. Overall chromatographic performance amongst the eight different materials was similar (peak shape, width), with the most notable differences observed in the retention time and elution order of peptides.

Chromatographic separation of 300 fmol enolase digest, on-column injection, on a 75µm ID PicoFrit column packed with 10 cm of Hypercarb Porous Graphitic Carbon. Hypercarb exhibits increase retention of polar analytes. The broad peaks and tailing indicate the peptide digest is too hydrophobic for this stationary phase.

FIGURE 6

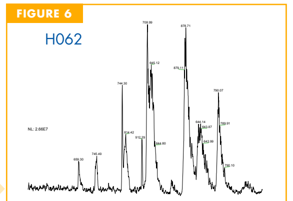
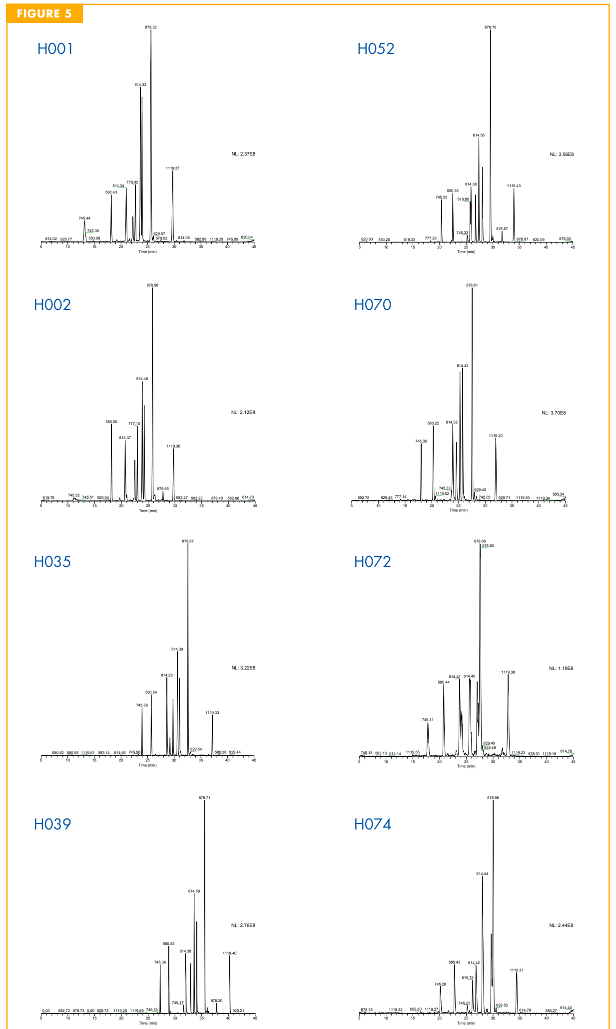


FIGURE 5



300 fmol On-column injection enolase digest separated on eight different reversed phase materials.

Conclusions

- Selectivity of different stationary phases demonstrated in the nanobore column format
- Separations using Porous Graphitic Carbon (Hypercarb) require further investigation
- Additional stationary phases for reversed phase separation in the nanobore column format will be evaluated

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