

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 quantitative 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

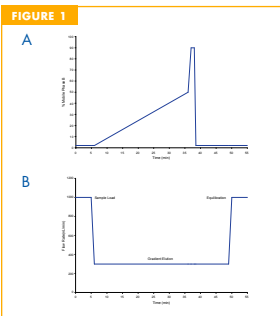
- 75 μm ID PicoFrit column with a 15 μm mobile frit; slurry packed to 10 cm with selected chemistries

Samples

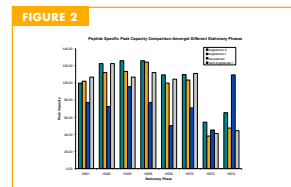
- Enolase digest (Waters MassPrep)
 - ~ 300 fmol/ μL in 0.1% formic acid
- Equimolar mix of four peptides
 - ~ 200 fmol/ μL in 0.1% formic acid/H₂O
 - Angiotensin I, 1296 Da
 - Angiotensin II, 1045 Da
 - Val¹-Angiotensin I, 1282 Da
 - Neurotensin, 1672 Da

Media Code	Actual Bed Length (mm)	Chemistry			Pressure	
		Brand Name	Particle Size (μm)	Pore Size (\AA)	Flow Rate (nL/min)	Mobile Phase
H001	100	ProteinPip	5	300	C18	1,168
H002	98	ProteinPip II	5	300	C18	1,168
H025	100	HALO	2.7	90	C18	3,122
H030	98	HALO-Pipetas	2.7	150	ES-C18	3,557
H052	102	BioBasic	5	300	C18	2,415
H052	99	Hypercarb	5	250	PGC	1,630
H070	100	HyperGOLD	5	175	C18	1,538
H072	100	HyperGOLD	5	175	PPP	1,440
H074	103	HyperGOLD	5	175	C18AG	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



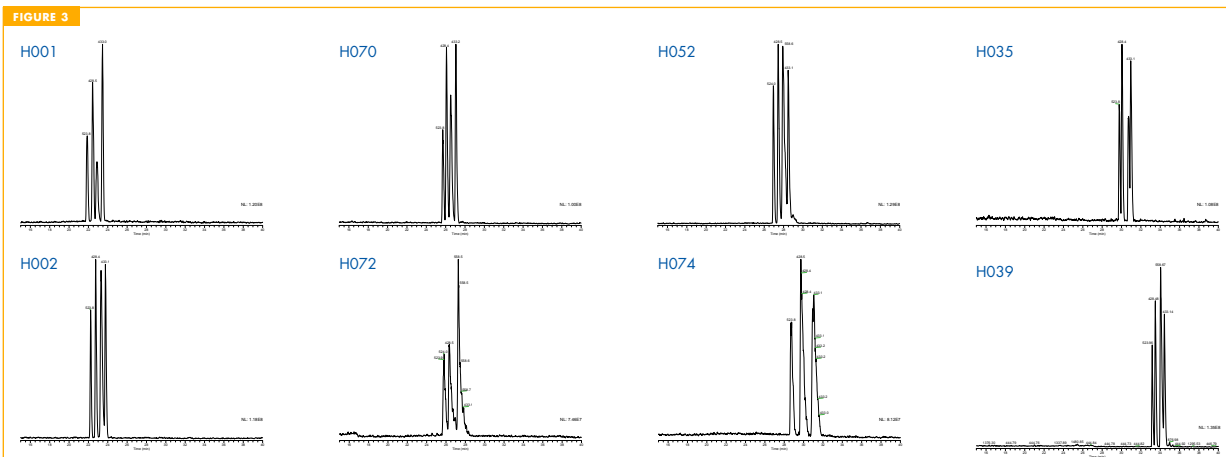
Gradient and flow rate profiles used for data collection. A) 55 min gradient profile B) flow rate profile



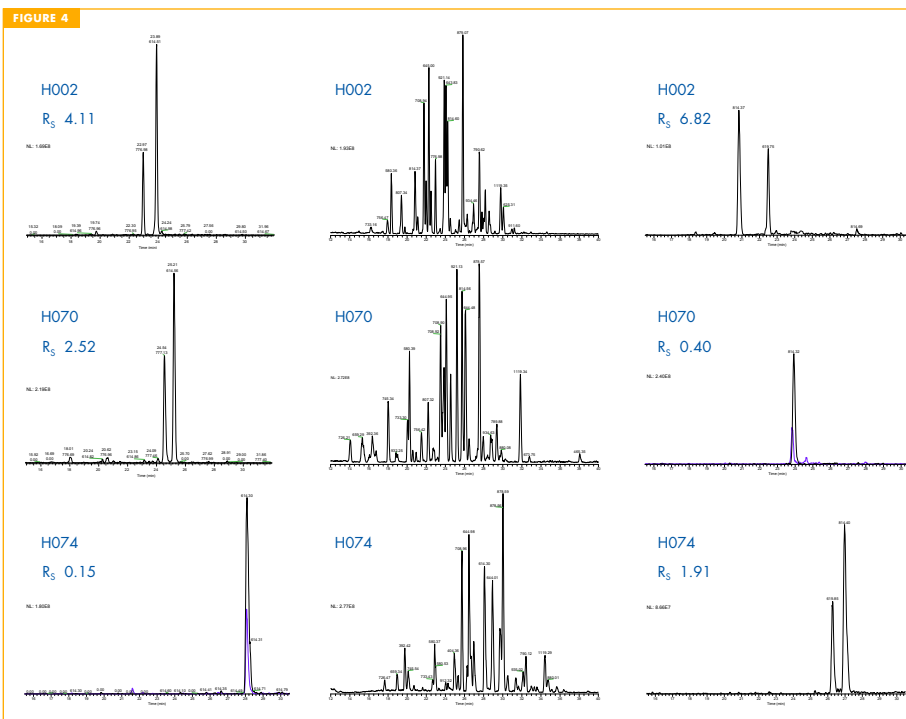
Plot of peptide specific peak capacity calculated for four standard peptides separated on a 75um ID, 10 cm PicoFrit column packed with eight different reversed phase materials

Stationary Phase	Peptide Name	m/z (Da)	Peptide Sequence	Apex RT (min)	Start RT (min)	End RT (min)	Area	Height (mm)	Peak Width (min)	Peak Capacity
H001	Angiotensin I	1296	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Angiotensin II	1045	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Val ¹ -Angiotensin I	1282	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Neurotensin	1672	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
H002	Angiotensin I	1296	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Angiotensin II	1045	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Val ¹ -Angiotensin I	1282	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Neurotensin	1672	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
H025	Angiotensin I	1296	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Angiotensin II	1045	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Val ¹ -Angiotensin I	1282	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Neurotensin	1672	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
H030	Angiotensin I	1296	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Angiotensin II	1045	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Val ¹ -Angiotensin I	1282	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Neurotensin	1672	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
H052	Angiotensin I	1296	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Angiotensin II	1045	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Val ¹ -Angiotensin I	1282	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Neurotensin	1672	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
H052	Angiotensin I	1296	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Angiotensin II	1045	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Val ¹ -Angiotensin I	1282	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Neurotensin	1672	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
H070	Angiotensin I	1296	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Angiotensin II	1045	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Val ¹ -Angiotensin I	1282	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Neurotensin	1672	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
H070	Angiotensin I	1296	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Angiotensin II	1045	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Val ¹ -Angiotensin I	1282	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Neurotensin	1672	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
H072	Angiotensin I	1296	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Angiotensin II	1045	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Val ¹ -Angiotensin I	1282	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Neurotensin	1672	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
H074	Angiotensin I	1296	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Angiotensin II	1045	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Val ¹ -Angiotensin I	1282	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79
	Neurotensin	1672	NDYKQK	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	199.79

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



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.



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

Chromatographic separation of 300 fmol enolase digest, on-column injection, on a 75um 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.

Stationary Phase	Apex RT (min)	Start RT (min)	End RT (min)	Area	Height (mm)	Peak Width (min)	Asymmetry
H001	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
H002	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
H025	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
H030	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
H052	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
H052	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
H070	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
H070	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
H072	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
H074	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+08	4.98E+07	0.30	1.00
	22.24	22.21	22.29	4.98E+0			