TN-1382

Reversed-Phase Selectivity for Peptide Mapping

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Introduction

Peptide mapping via reversed-phase liquid chromatography mass spectrometry (LC-MS) is primarily used to analyze and confirm a protein's amino acid sequence, including the identification and localization of post-translational or synthetic modifications. This level of characterization is part of the critical quality attributes (CQA) assessment required for biopharmaceutical products since unexpected modifications can impact the stability and function of a biotherapeutic drug. Protein characterization via peptide mapping is challenging because some peptides can have very similar physiochemical properties. For this reason, selecting a fit-for-purpose chromatographic column(s) must be carefully considered. In this technical note, the performance of 13 reversed-phased columns belonging to three distinct column chemistry families were evaluated. Both a mixture of seven peptides, commonly used as standards in proteomics LC-MS analyses, and a digested sample of the NIST monoclonal antibody reference material (NIST mAb) were analyzed.

Columns

- Kinetex™ family - core-shell particle technology designed for improved efficiency and resolution.
- Luna™ Omega family - fully porous, silica particle technology designed for enhanced inertness and reproducibility.
- Biozen™ family - BioTi™ bio-inert hardware to maximize selectivity and sensitivity for biomolecules

Samples

1. **Peptide Mixture:** Seven peptides with a broad range of polarities and isoelectric points (pI) (**Table 1**).
2. **NIST mAb**: Reduced and alkylated National Institute of Standards and Technology monoclonal antibody reference material, RM 8671 (**Table 2**).

Table 1. Peptide mixture with corresponding sequence, molecular weight, isoelectric point (pI) and grand average of hydropathicity (GRAVY) score.

Peptide Name	Peptide Sequence	Molecular Weight (g/mol)	pI	GRAVY Score
RASG-1	RGDSPASSKP	1000.49	8.75	-1.61
Angiotensin frag. 1-7	DRVYIHP	898.46	6.74	-0.77
Bradykinin	RPPGFSPFR	1059.56	12.00	-1.04
Angiotensin II	DRVYIHPF	1045.53	6.74	-0.33
Angiotensin I	DRVYIHPFHL	1295.67	6.92	-0.20
Renin substrate	DRVYIHPFHLLVYS	1757.92	6.92	0.28
Enolase T35	WLTGPQLADLYHSLMK	1871.96	6.74	-0.06

LC Conditions

Mobile Phase: **A**: 0.2% Formic Acid in Water
B: 0.2% Formic Acid in (80:20) Acetonitrile:Water

Sample:	Peptide Mixture		Digested NIST mAb	
Gradient Used:	**Time (min)**	**%B**	**Time (min)**	**%B**
	0	2	0	0.5
	2	2	2	0.5
	15	65	62	50
	15.1	85	65	50
	17.1	85	66	95
	17.2	2	68	0.5
	28	2	80	0.5

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LC Conditions

Sample:	Peptide Mixture	Digested NIST mAb
Flow Rate:	0.200 µL/min	0.300 µL/min
Injection Volume:	10 µL	5 µL
Concentration:	1.5 ng/ µL	500 ng/ µL
Temperature:	65 °C	
LC System:	Agilent® 1260	
Detection:	ESI Positive	
Detector:	SCIEX™ ZenoTOF 7600	

Mass Spectrometry Conditions

TOF MS Parameters

Ion source gas 1 (PSI):	50
Ion source gas 2 (PSI):	50
Curtain gas: (PSI)	35
CAD gas (PSI):	7
Source temperature (C):	350
Spray Voltage (V):	3500
TOF start mass (mz):	200
TOF stop mass (mz):	2000
Accumulation time (ms):	0.1
Declustering potential (V):	20
Collision energy (V):	10
Time bins to sum:	8

EAD Parameters

IDA criteria:	Peptide
Maximum ions:	6
Intensity threshold:	100
Charge state:	2-10
Isotope to select:	Most Intense
Exclude time:	6s after 2 occurrences
Dynamic ETC :	True
TOF start mass:	100
TOF stop mass:	3000
Accumulation time:	0.09
Q1 resolution:	Unit
Zeno trap:	True
Electron beam current:	5500
Electron KE:	7 eV

Analysis Software

Sciex Biologics Explorer was used for peptide identification. Skyline (MacCoss Lab Software) was used for data visualization.

List of Columns Evaluated and Part Numbers

Column	Dimensions	Part No.
Kinetex 2.6 µm C18	150 x 2.1 mm	00F-4462-AN
Kinetex 2.6 µm XB-C18	150 x 2.1 mm	00F-4496-AN
Kinetex 2.6 µm Polar C18	150 x 2.1 mm	00F-4759-AN
Kinetex 2.6 µm PS C18	150 x 2.1 mm	00F-4780-AN
Kinetex 2.6 µm Biphenyl	150 x 2.1 mm	00F-4622-AN
Kinetex 2.6 µm Phenyl-Hexyl	150 x 2.1 mm	00F-4495-AN
Kinetex 2.6 µm F5	150 x 2.1 mm	00F-4723-AN
Luna Omega 3 µm C18	150 x 2.1 mm	00F-4784-AN
Luna Omega 3 µm Polar C18	150 x 2.1 mm	00F-4760-AN
Luna Omega 3 µm PS C18	150 x 2.1 mm	00F-4758-AN
Biozen 2.6 µm Peptide XB-C18	150 x 2.1 mm	00F-4768-AN
Biozen 1.6 µm Peptide Polar C18*	150 x 2.1 mm	00F-4782-E0
Biozen 3 µm Peptide PS C18	150 x 2.1 mm	00F-4768-AN

Peak Capacity on Peptide Mix

Peak capacity (PC) is a measure of how many peaks can be separated within a given analysis time and serves as an indicator of the column's separation power. Higher peak capacity values indicate better separation of analytes which will consequently result in better data quality. We first used the Peptide Mixture to investigate the peak capacity of the Kinetex™, Luna™ Omega, and Biozen™ column families. The average peak capacity was calculated for each column and plotted in **Figure 1.** Looking at the results in the context of particle morphology, columns with core-shell particles provided the best peak capacities. The highest performing column chemistry within each family was the Polar C18 **(Figure 1 and 3).** The high peak capacities seen for core-shell particles are not unexpected, as these were designed to provide faster mass transfers of analytes in and out of the stationary phase when compared to particles of the similar size but made of fully-porous silica.

Figure 1. Average peak capacity of all screened columns.

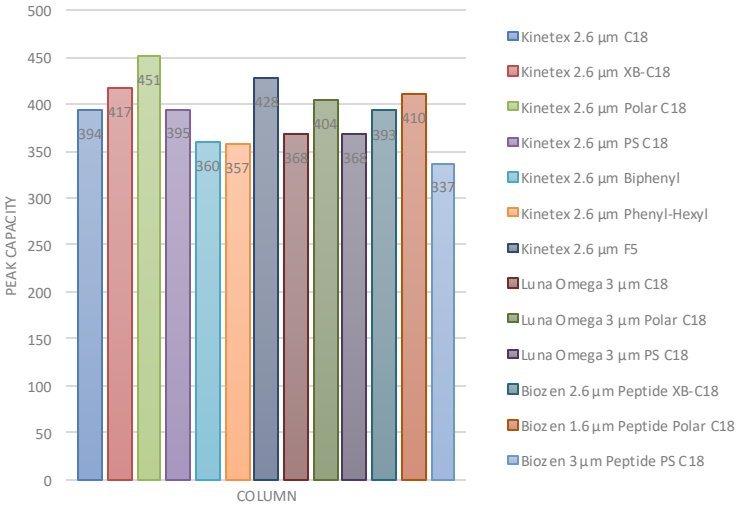
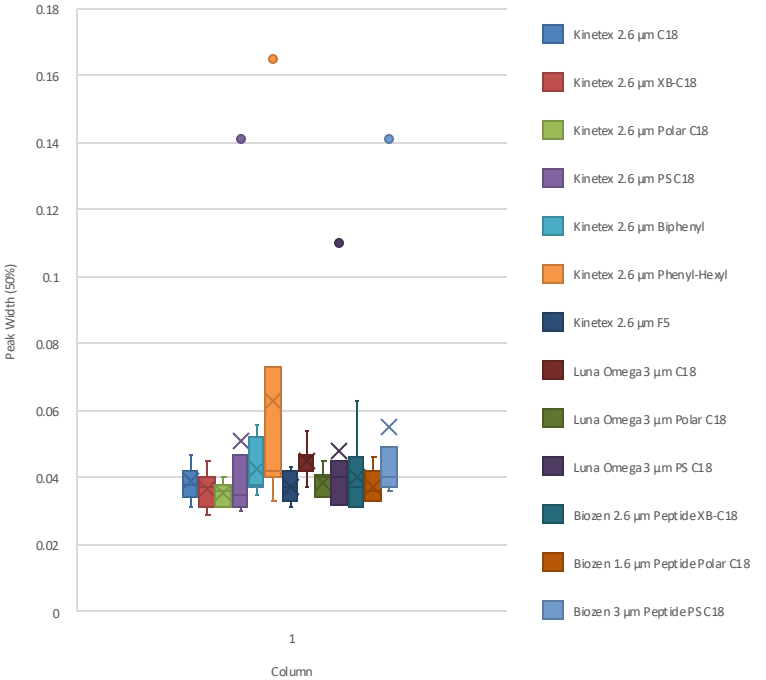


Figure 2. Box plots showing the distribution of peak width at 50 % base width observed for each column evaluated.



An additional factor that contributes to the higher efficiencies observed for core-shell particles relates to their very narrow particle size distribution, thus reducing band broadening. Regarding the increased capacity seen for columns of polar nature, this could be explained by the fact that most of the peptides in our mixture are hydrophilic. Taking a closer look at the distribution of peak width by column type in **Figure 2**, we can observe that the PS chemistry columns across the three families had RASG-1
as an outlier with half-height peak widths > 0.1. RASG-1 peptide has a GRAVY score of -1.61 and 2 basic residues which carry positive charges (**Table 1**), making it the most hydrophilic peptide in this peptide mixture. The hydrophilic and charged nature of this peptide results in increased repulsion with the positively charged PS surface causing RASG-1 to elute much earlier and with poor peak shape. An example of RASG-1 poor peak shape and lower retention can be observed when comparing **Figure 3 and 4**. Considering all seven peptides, the columns with PS C18 chemistry seem to have lower peak capacities than expected. However, this observation is biased due to the outlier caused by the low retention and broader peak shape seen for basic peptide RASG-1 . If this peptide were to be removed, we would see peak capacities of 442, 402 and 373, for Kinetex PS-C18, Luna Omega PS-C18 and Biozen Peptide PS-C18, respectively.

Looking at the phenyl chemistry columns tested, Kinetex Phenyl-Hexyl and Biphenyl chemistries showed the lowest peak capacity, opposite to what was observed for Kinetex F5. This trend could be explained by the fact that even though all 3 columns offer π-π aromatic and polar interactions for selectivity, only F5 has strong H-bonding capabilities. In this evaluation, acetonitrile was selected as the organic solvent for testing to significantly hinder π-π interactions, thus reducing the additional selectivity these columns could offer.

Figure 3. TIC of the seven-peptide mixture on a Kinetex 2.6 µm Polar C18 column.

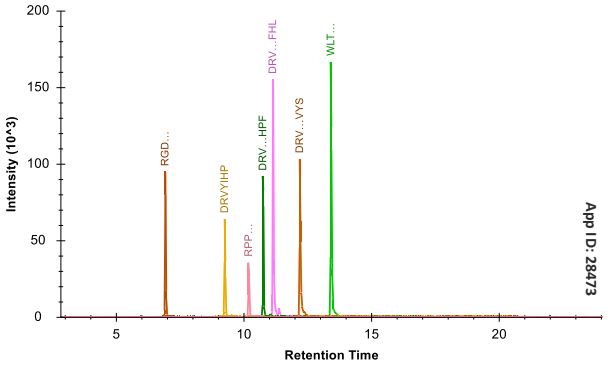
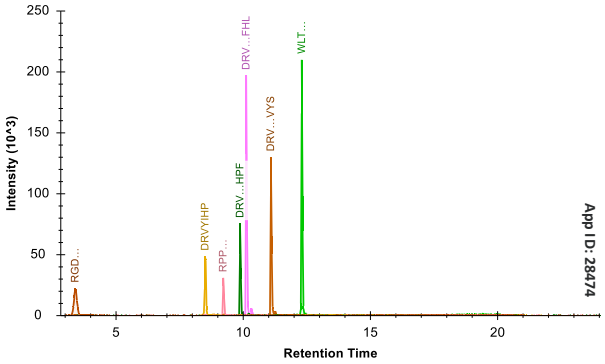


Figure 4. TIC of the seven-peptide mixture on a Biozen µm 3 µm Peptide PS C18. Retention time in minutes.



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Peptides From NIST mAb Digestion

Furthering on the insights gained on peak capacities estimated with a peptide mixture, a deeper analysis was done by evaluating the chromatographic performance for each of the 13 columns using a more complex tryptic digest mixture of the NIST mAb. The tryptic digestion process of the NIST mAb generated over 50 peptide fragments, 3 to 58 amino acids in length. **Figure 5** shows the NIST mAb heavy and light sequences, including cleavage sites. Several of these are considered critical quality attributes (CQAs) of the protein's structure and must be fully characterized, which is a challenge because these are often present in low abundances and may differentiate by only small modifications or isomerism. To explore how differences in physicochemical properties affect column performance, a selection of 52 peptides (**Table 3**) were categorized based on: (i) their content of polar uncharged, basic and acidic charged, and hydrophobic residue side chains, (ii) their length (with long peptides being defined as greater than 20 amino acids), (iii) the presence of deamidated N and Q residues, (iv) the presence of glycosylation. Example TIC for each column are shown in **Figure 6**.

Figure 5. Amino acid sequences of the Heavy and Light Chains of NIST mAb with Lys and Arg Trypsin cleavage sites highlighted in red.

Heavy Chain (HC) Sequence

pQVTLRESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIRQPPGKALEWLADIWWDDKKHYNPSLKDRLTISKDT
SKNQVVLKVTNMDPADTATYYCARDMIFNFYFDVWGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVK
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKRVEPKSCDKTH
TCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTY
RVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDI
AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

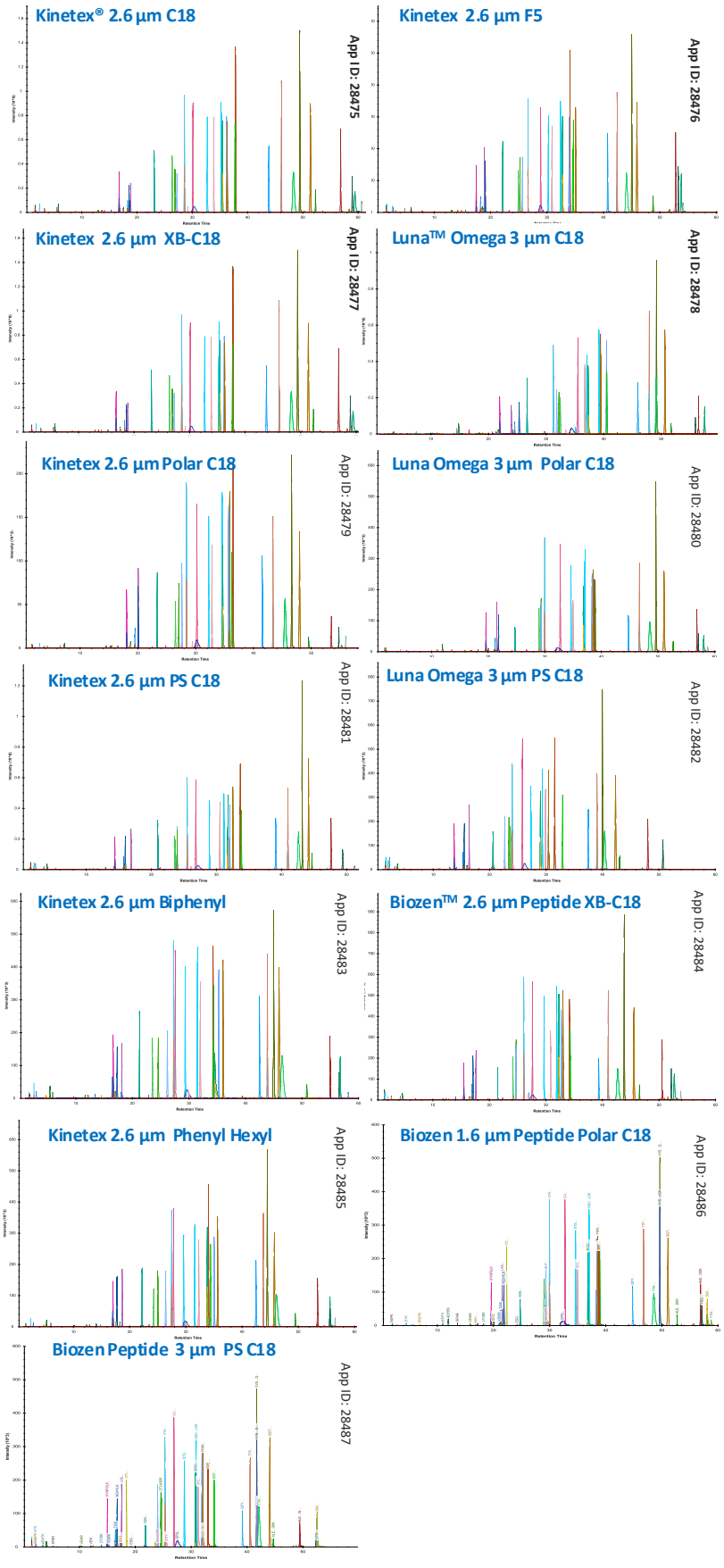
Light Chain (LC) Sequence

DIQMTQSPSTLSASVGDRVTITCSASSRVGYMHWYQQKPGKAPKLLIYDTSKLASGVPSRFSGSGSGTEFTLTISSLQ
PDDFATYYCFQGSGYPFTFGGGTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQS
GNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Table 2. NIST mAb peptides used for chromatographic calculations.

Component Name	Sequence	Length	Modification	PI	GRAVY
HC 321-323	EYK	3		0	0
LC 29-38	VGYMHWYQQK	C		8.48	-1.19
LC 207-210	SFNR	4		9.47	-1.50
LC 145-158	VQWK	4		8.72	-1.03
HC 46-58	ALEWLADIWWDDK	13		6.10	-0.05
HC 296-304 G0F	EEQYN(G0F)STYR	14	G0F (HexNAc)4 (Hex)3 (Fuc)		-2.51
HC 296-304 G2F	EEQYN(F2F)STYR	14	G2F (HexNAc)4 (Hex)5 Fuc		
LC 61-102	FSGSGSGTEFTLTISSLQPDDFATYYCFQGSGYPFTFGGGTK	42	Carbamidomethyl C	4.05	-0.18
HC 278-291	FNWYVDGVEVHNAK	14		4.17	-0.12
HC 46-58 Miss	ALEWLADIWWDDKK	14		4.05	-0.38
LC 183-187	ADYEK	5		4.37	-2.08
HC 100-124	DMIFNFYFDVWGQGTTVTVSSASTK	25		4.14	-0.10
HC 374-395	GFYPSDIAVEWESNGQPENNYK	22		4.05	-1.26
HC 396-412	TTPPVLDSDGSFFLYSK	17		4.21	0.13
HC 84-124	VTNMDPADTATYYCARDMIFNFYFDVWGQGTTVTVSSASTK	41	Carbamidomethyl C	8.75	0.22
HC 60-66	HYNPSLK	7		9.70	-1.80
HC 74-124	DTSKNQVVLKVTNMDPADTATYYCARDMIFNFYFDVWGQGTTVTVSSASTK	51	Carbamidomethyl C	8.75	0.58
HC 420-442	WQQGNVFSCSVMHEALHNHYTQK	23	Carbamidomethyl C	7.01	-0.79
HC 59-66	KHYNPSLK	8		10.20	-1.80
LC 126-141	SGTASVVCLLNNFYPR	16	Carbamidomethyl C	7.94	0.38
LC 45-52	LLIYDTSK	8		5.83	0.24
LC 108-125	TVAAPSVFIFPPSDEQLK	18		4.37	0.24
HC 151-213	DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHK	58	Carbamidomethyl C	7.94	0.70
HC 348-358	EPQVYTLPPSR	11		6.90	-1.01
HC 125-136	GPSVFPLAPSSK	12		4.21	0.12
LC 169-182	DSTYSLSSTLTLSK	14		5.83	-0.24
HC 305-320	VVSVLTVLHQDWLNGK	16		4.53	-2.51
LC 190206	VYACEVTHQGLSSPVTK	17	Carbamidomethyl C	6.71	0.02
HC 292-295	TKPR	4		5.32	-0.46
HC 342-347	AKGQPR	6		11.42	-2.02
HC 217-221	RVEPK	5		5.99	0.14
HC 218-221	RVEPK	5		8.75	-1.86
LC 103-107	VEIKR	5		8.72	-0.64
HC 6-13	ESGPALVK	8		9.75	-0.14
HC 330-337	ALPAPIEK	8		6.90	0.16
LC 53-60	LASGVPSR	8		9.75	0.21
HC 67-73	DRLTISK	7		8.60	-1.50
HC 137-150	STSGGTAALGCLVK	14	Carbamidomethyl C	8.75	0.09
LC 103-106	VEIK	4		5.97	0.33
HC 326-329	VSNK	4		10.1	-1.00
HC 338-341	TISK	4		6.05	0.16
HC 252-291	TPEVTCVVVDVSHEDPEVK	19	Carbamidomethyl C	5.84	0.10
HC 359-363	EEMTK	5		4.53	-1.94
HC 413-417	LTVDK	5		5.84	-0.02
HC 69-73	LTISK	5		10.1	0.58
LC 1-18	DIQMTQSPSTLSASVGDR	18		4.21	-0.49
HC 1-5 (pQ)	pQVTLR	6	Pyroglutamic Acid Q N-TERM	9.75	-0.14
HC 78-83	NQVVLK	6		4.69	-0.23
HC 252-258	DTLMISR	7		5.97	-1.20
LC 149-168	VDNALQSGNSQESVTEQDSK	20		4.05	-1.29
LC 19-28	VTITCSASSR	10	Carbamidomethyl C	8.22	0.47
HC 364-373	NQVSLTCLVK	10	Carbamidomethyl C	8.22	0.61

Figure 6. TIC of peptides from Trypsin digested NISTmAb standard, separated using a wide variety of Phenomenex columns. Retention Times are in minutes.



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Polar Peptides

To assess the effect of peptide polarity and peak capacity, a group of NIST mAb peptides categorized as "polar" were considered. This category requires the peptides to have at least 35 % of their amino acid residues being of polar uncharged nature and includes two peptides that contain either GF0 or GF2, both highly polar glycosylation modifications (**Table 3**). Within the Kinetex™ family, Polar C18 provided the highest peak capacity (**Figure 7**) and was able to completely resolve glycosylated peptides (**Figures 8).** Kinetex F5, Phenyl-Hexyl, and PS C18 chemistries still proved beneficial for peptides that contained a higher aromatic and basic content. Interestingly, the Luna™ Omega family provided the lowest chromatographic resolution for these polar peptides with Luna Omega 3 µm Polar C18 having the highest peak capacity within the its family.

Figure 7. Average peak capacity for polar peptides of all screened columns.

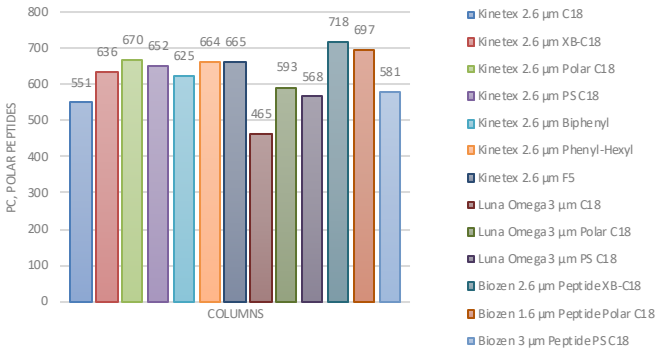


Table 3. Neutral polar peptides used to calculate peak capacities in **Figure 7**.

Component Name	Sequence	Length	%Polar
LC 19-28	VTITCSASSR	10	60%
LC 169-182	DSTYSLSSTLTLSK	14	57%
LC 149-168	VDNALQSGNSQESVTEQDSK	20	50%
LC 1-18	DIQMTQSPSTLSASVGDR	18	50%
HC 100-124	DMIFNFYFDVWGQGTTVTVSSASTK	25	40%
HC 151-213	DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHK	58	40%
HC 74-124	DTSKNQVVLKVTNMDPADTATYYCARDMIFNFYFDVWGQGTTVTVSSASTK	51	39%
HC 84-124	VTNMDPADTATYYCARDMIFNFYFDVWGQGTTVTVSSASTK	41	39%
LC 61-102	FSGSGSGTEFTLTISSLQPDDFATYYCFQGSGYPFTFGGGTK	42	36%
HC 396-412	TTPPVLDSDGSFFLYSK	17	29%
HC 125-136	GPSVFPLAPSSK	12	25%
HC 296-304	EEQYN(G0F)STYR		
HC 296-304	EEQYN(F2F)STYR		

Figure 8. EIC of EEQYNSTYR peptide with G0F and G2F Glycans at N5 and nearby peptides on a Kinetex 3 µm Polar C18 (**Right**) and on a Kinetex 2.6 µm Biphenyl (**Left**).

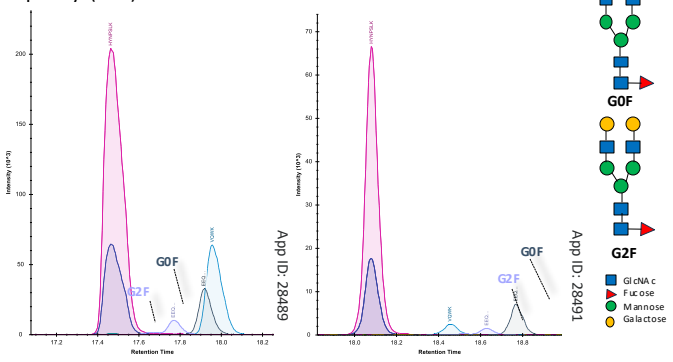
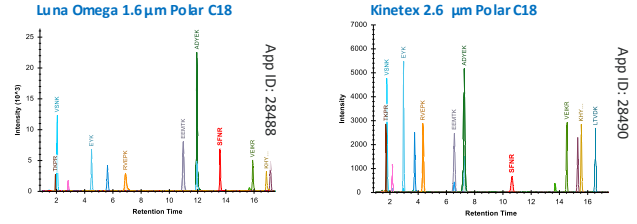
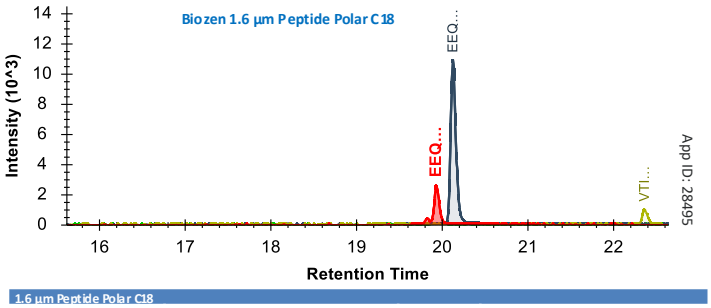


Figure 9. Example of the high retentivity of the Luna Omega column on hydrophilic peptides.



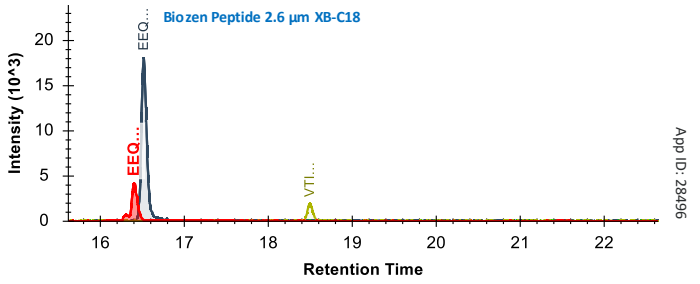
Despite the lower peak capacities that resulted from the Luna Omega columns, Luna Omega Polar C18 excelled at delivering better peak shape for early eluting polar peptides when compared to Kinetex. These highly hydrophilic peptides are difficult to retain using typical C18 phases, but with Luna Omega Polar C18, increased retention is observed due to its thermally modified particle that eliminates micropores optimizing the available surface area for interaction with the polar end-capped surface. This is demonstrated in **Figure 9**, where Luna Omega 3 µm Polar C18 clearly provides higher retention for hydrophilic peptides. As expected, this trend is also observed for our Biozen™ Peptide Polar C18 column, which provided good peak capacity with enhanced retention (see **Figure 10**) and improved peak shape of early eluting peaks. Interestingly, in this case, the Peptide XB-C18 chemistry was the best performing, which suggests that the bioinert hardware contributes to the peak capacity increase. The Biozen 1.6 µm Peptide Polar C18 column shows improved peak retention of glycosylated peptides which, combined with its polar selectivity and the higher efficiency of a 1.6 µm particle, successfully achieved baseline resolution between the G2F and GF0 glycosylated forms of EEQYN*STYR. Thus, making this column a powerful tool for the separation of highly polar and glycosylated peptides. Extracted ion chromatograms (EIC) comparing these glycosylated peptides within the Biozen column family are shown in **Figure 10**.

Figure 10. EICs of polar peptides EEQYNSTYR with G0F and G2F Glycan modifications at N5 and VTITCSASSR on a Biozen Peptide Polar C18 (**Top**), XB-C18 (**Middle**) and PS (**Bottom**) columns and respective Resolution (Rs) values.



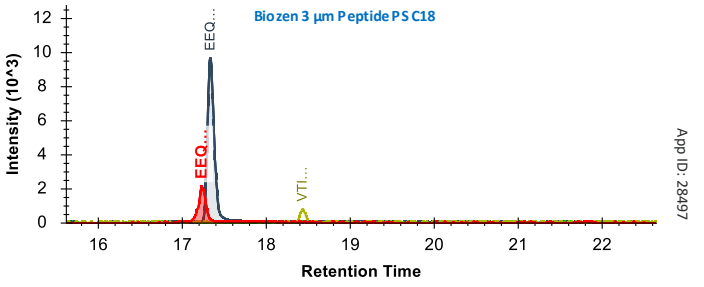
1.6 µm Peptide Polar C18

Peptide	RT	Peak Width	Resolution
EEQYN(G2F)STYR	19.95	0.16	
EEQYN(GF0)STYR	20.15	0.21	1.07
VTITCSASSR	22.39	0.22	10.57



Peptide 2.6 µm XB-C18

Peptide	RT	Peak Width	Resolution
EEQYN(G2F)STYR	16.45	0.21	
EEQYN(GF0)STYR	16.54	0.22	0.44
VTITCSASSR	18.50	0.22	8.88



Peptide 3 µm PS C18

Peptide	RT	Peak Width	Resolution
EEQYN(G2F)STYR	17.24	0.36	
EEQYN(GF0)STYR	17.38	0.28	0.44
VTITCSASSR	18.45	0.22	4.28

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Acidic Peptides

For this analysis, NIST mAb acidic peptides that have greater than or equal to 29% of their sequence containing acidic residues (D, E) (**Table 4**) were used to evaluate peak capacities (**Figure 11**) across the column families. In the case of acidic peptides, the top performers were Kinetex™ 2.6 µm PS C18 closely followed by Biozen™ 2.6 µm XB-C18. Overall, the core-shell capabilities of these columns offer advantages in terms of efficiency and speed when compared to fully porous particles. This translates to shorter run times and improved peak shape. Within the Kinetex family, the PS C18 (2.6 µm) column had greater resolution between two acidic peptides while XB-C18 (2.6 µm) maintained baseline resolution with increased retention. Notably, the peak capacity XB-C18 increased by approximately 7% when coupled with bioinert hardware. Therefore, the PS-C18 chemistry provides good retention of acidic peptides while the bioinert hardware improves its reproducibility.

Figure 11. Peak capacity for acidic peptides on the Kinetex family of columns.

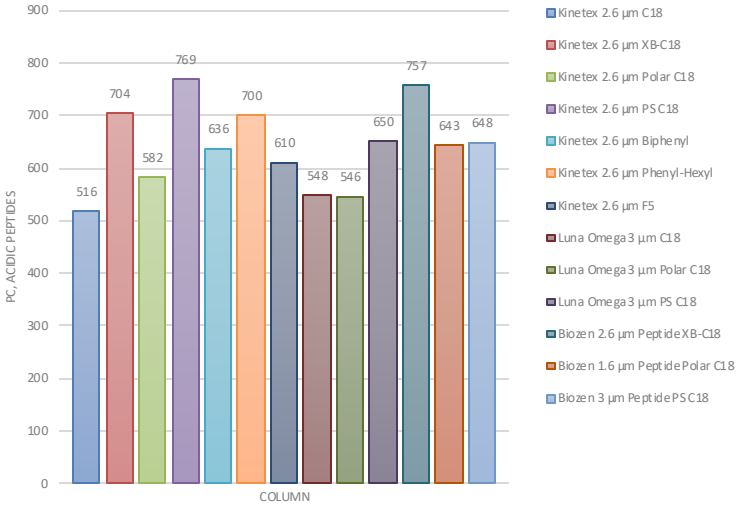
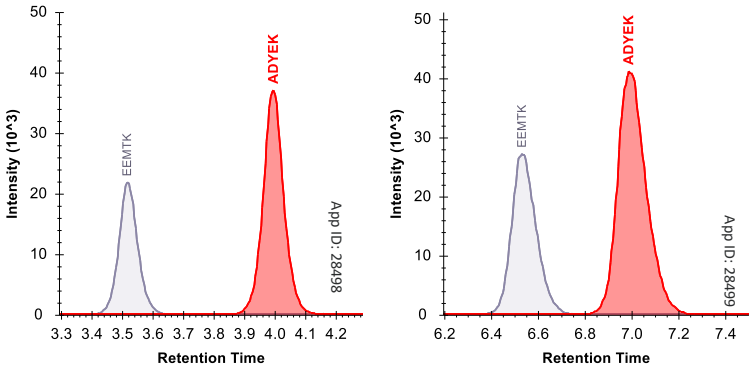


Table 4. Acidic peptides used to calculate peak capacities in **Figure 11**.

Component Name	Sequence	Length	% Acidic
HC 359-363	EEMTK	5	40%
LC 183-187	ADYEK	5	40%
HC 321-323	EYK	3	33%
HC 46-58	ALEWLADIWWDDK	13	31%
HC 46-59	ALEWLADIWWDDKK	14	29%
HC 252-291	TPEVTCVVVDVSHEDPEVK	19	26%
LC 103-106	VEIK	4	25%

Figure 12. Example of acidic peptide resolution on a Kinetex 2.6 µm PS column (**Left**) and a Kinetex 2.6 µm C18 column (**Right**).



Kinetex 2.6 µm PS Column

Peptide Sequence	Peak Width (PW)	PW 50 %	RT	Resolution
EEMTK	1.17	0.068	3.52	
ADYEK	0.96	0.070	4.00	2.17

Kinetex 2.6 µm C18 Column

Peptide Sequence	Peak Width (PW)	PW 50 %	RT	Resolution
EEMTK	1.28	0.120	6.54	
ADYEK	1.14	0.130	7.00	2.09

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Basic Peptides

The Kinetex™ PS C18 column's positively charged surface modification enhances selectivity and improves peak shape not only for acidic peptides, but also for basic peptides under reversed phase conditions. Additional columns that demonstrated efficient basic peptide separation are Kinetex 2.6 µm XB-C18, Biozen Peptide 2.6 µm XB-C18 and Kinetex 2.6 µm F5 columns. (**Figures 13**). An example of the resolving power of the Biozen 3µm Peptide PS and on the Kinetex 2.6 µm PS columns is shown in **Figure 14.** While both Biozen Peptide and Kinetex versions of the PS chemistry provided excellent resolution for basic peptides, the Kinetex provided the highest due to the higher efficiency core-shell particle morphology. Basic peptides used to calculate peak capacities and peak asymmetries are shown in **Table 5**.

Figure 13. Peak capacity for basic peptides on the Kinetex family of columns.

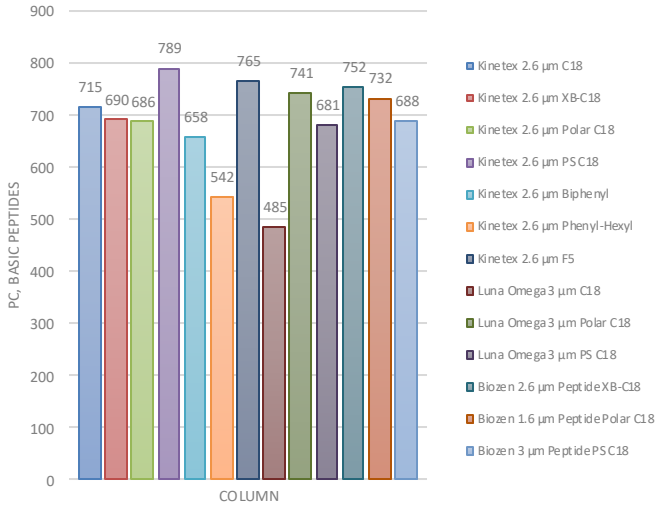
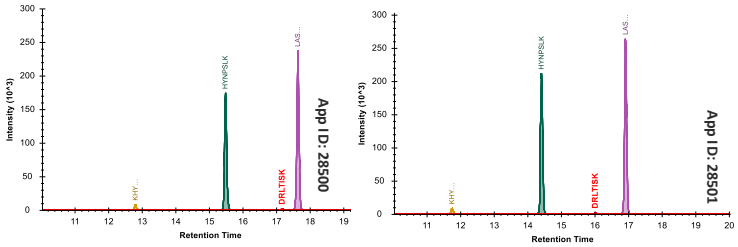


Table 5. Example of basic peptides used to calculate peak capacities in **Figure 13**.

Component Name	Sequence	Length	% Basic
HC 342-347	AKGQPR	6	67%
HC 59-66	KHYNPSLK	8	50%
HC 326-329	VSNK	4	25%
HC 69-73	LTISK	5	20%
HC 1-5 (pQ)	pQVTLR	6	17%
LC 53-60	LASGVPSR	8	38%
HC 6-13	ESGPALVK	8	38%
HC 60-66	HYNPSLK	7	43%
LC 207-210	SFNR	4	25%

Figure 14. Example of basic peptide resolution on a Biozen 3 µm Peptide PS column (**Left**) and a Kinetex 2.6 µm PS (**Right**).



Biozen Peptide 2.6 µm PS C18

Peptide Sequence	Peak Width (PW)	RT	Resolution
KHYNPSLK	0.24	12.85	
HYNPSLK	0.23	15.50	11.27
DRLTISK	0.22	17.16	7.45
LASGVPSR	0.22	17.64	2.21

Kinetex 2.6 µm PS C18

Peptide Sequence	Peak Width (PW)	RT	Resolution
KHYNPSLK	0.21	11.76	
HYNPSLK	0.21	14.42	12.57
DRLTISK	0.23	16.02	7.16
LASGVPSR	0.21	16.92	4.08

A frequent issue when working with basic peptides is peak tailing due to unwanted secondary interactions with the stationary phase. Peak Asymmetry (PA) is a measurement of peak shape, where values lower than 1 indicate peak fronting and values higher than 1 indicate peak tailing. Here, PA values were calculated for all columns and are shown in **Figures 14** and **Table 6.** Looking both at the median PA values per column and the PA distribution obtained for the basic peptides under study, the PS C18 chemistry offers a clear advantage in peak shape by reducing peak tailing. **Figure 15** shows a comparison of peak shape for the basic peptide KHYNPSLK when using PS C18 and XB-C18 chemistries for both Kinetex™ and Biozen™ Peptide columns, and when using PS 18 and C18 for Luna™ Omega columns.

Figure 14. Peak asymmetry distribution for basic peptides on the three families of columns.

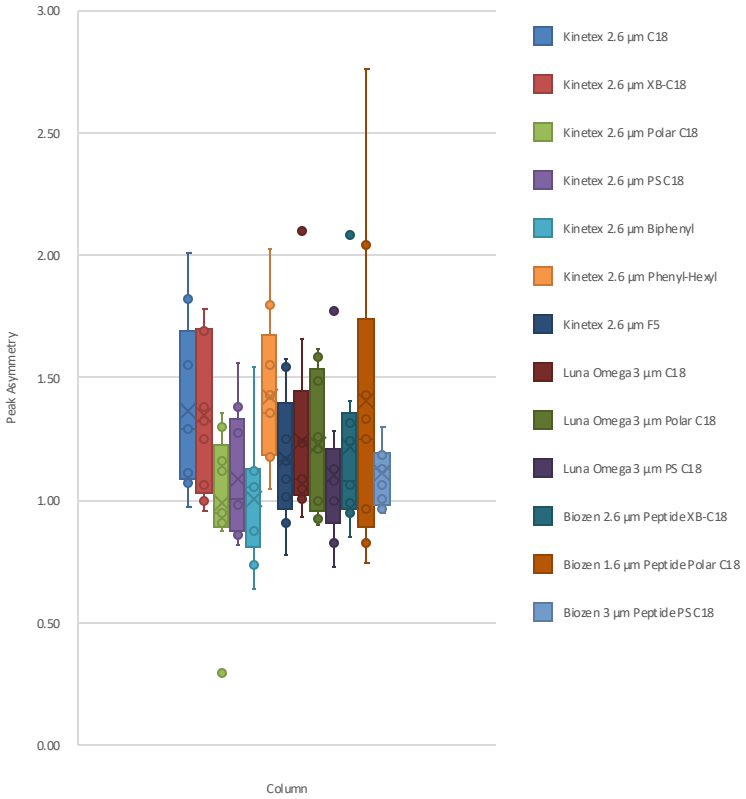
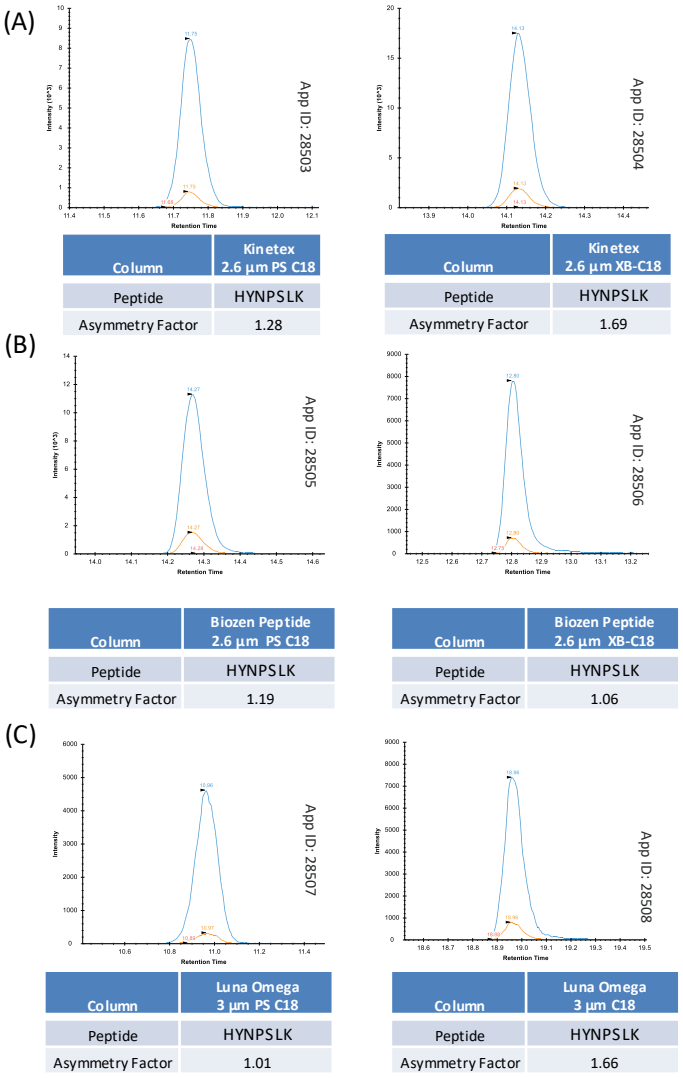


Table 6. Average peak asymmetry for basic peptides in all column families.

Column	TF (Ave), Acidic Peptides
Kinetex 2.6 µm C18	1.18
Kinetex 2.6 µm XB-C18	1.24
Kinetex 2.6 µm Polar C18	1.05
Kinetex 2.6 µm PS C18	1.05
Kinetex 2.6 µm Biphenyl	1.10
Kinetex 2.6 µm Phenyl-Hexyl	1.13
Kinetex 2.6 µm F5	1.14
Luna Omega 3 µm C18	1.26
Luna Omega 3 µm Polar C18	1.17
Luna Omega 3 µm PS C18	1.05
Biozen 2.6 µm Peptide XB-C18	1.49
Biozen 1.6 µm Peptide Polar C18	1.22
Biozen 3 µm Peptide PS C18	1.15

Figure 15. Comparison of EICs of basic peptide HYNPSLK on: **(A)** a Kinetex PS C18 and XB-C18 columns, (**B**) Biozen Peptide PS C18 and XB-C18 columns and **(C)** Luna Omega PS C18 and C18 columns.



Long Peptides

The effect of peptide length in peak capacity was assessed by looking at peptides longer than 20 residues or greater than 2000 Da. **Table 7** shows the list of large/long peptides used to calculate peak capacities. As shown in **Figure 16,** Biozen™ 2.6 µm Peptide XB-C18 had the highest peak capacity for long peptides while the lowest performing column was the fully porous Luna™ Omega 3 µm C18. The observed difference in performance between these two C18 phases can be explained by two main characteristics of the Biozen Peptide XB-C18 chemistry: (i) the presence of the di-isobutyl side chains that provide steric hindrance reduction allowing for more effective interactions of longer peptides with the stationary phase, and (ii) the core-shell particle morphology that minimizes analyte diffusion resulting in narrower peaks. Overall, core-shell media outperformed fully-porous (**Figure 16**), except for the Biozen 1.6 µm Peptide Polar C18 column due to its smaller particle size that provides higher resolution and separation efficiency. Additionally, when comparing Biozen Peptide XB-C18 and Kinetex XB-C18, an increase in peak capacity was clearly observed suggesting that the BioTi™ hardware plays a role on recovery and reproducibility of longer peptides. **Figure 17** shows an example of the resolving power for the largest two NIST peptides of the Biozen Peptide 2.6 µm XB-C18 column in comparison to the Luna Omega 3 µm C18 column. While Luna Omega provides higher retention, the Biozen Peptide XB-C18 achieves greater separation.

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Figure 16. Peak capacity for long peptides across Kinetex, Luna Omega, and Biozen column families.

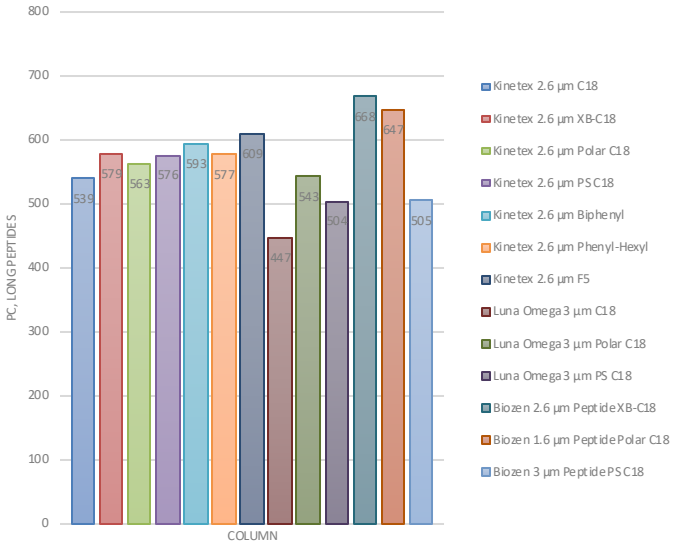
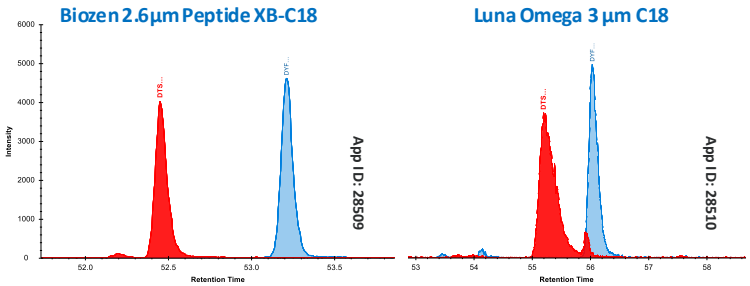


Table 7. Example of long peptides used to calculate peak capacities in **Figure 16**.

Sequence	Length	% Basic	MW	% Aromatic	% ACIDIC
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHK	58	5%	6128.004	10%	3%
DTSKNQVVLKVTNMDPADTATYYCARDMIFNFYFDVWGQGTTVTVSSASTK	51	8%	5685.663	14%	10%
FSGSGSGTEFTLTISSLQPDDFATYYCFQGSGYPFTFGGGTK	42	2%	4424.963	21%	7%
VTNMDPADTATYYCARDMIFNFYFDVWGQGTTVTVSSASTK	41	5%	4573.044	17%	10%
DMIFNFYFDVWGQGTTVTVSSASTK	25	4%	2800.294	20%	8%
WQQGNVFSCSVMHEALHNHYTQK	23	17%	2743.227	13%	4%
GFYPSDIAVEWESNGQPENNYK	22	5%	2543.113	18%	18%
VDNALQSGNSQESVTEQDSK	20	5%	2134.950	0%	20%

Figure 17. Kinetex family of column chemistries effect on peak capacity when using long peptides (>40 AAs). Retention times in minutes.



Peptide	RT (min)	Peak width	Resolution
DTSKNQVVLKVTNMDPADTATYYCARDMIFNFYFDVWGQGTTVTVSSASTK	52.49	0.25	
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHK	53.24	0.26	108.29

Peptide	RT (min)	Peak width	Resolution
DTSKNQVVLKVTNMDPADTATYYCARDMIFNFYFDVWGQGTTVTVSSASTK	55.40	0.84	
DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHK	56.13	0.55	5.06

Deamidation

Choosing a column for comprehensive characterization of complex deamidation profiles, often encountered during stability studies, is critical. The co-existence of multiple aspartic (Asp) and isoaspartic acid (IsoAsp) isomers poses an analytical challenge to chromatographic separations.

In this study, **Figure 18** shows the ability of Luna Omega 3 µm PS C18 column to fully resolve the isobaric deamidated forms of the VVSVLTVLHQDWL**N**GK peptide. The improvement in resolution between the non-deamidated and deamidated peptide forms suggests that the ion exchange interactions offered by the positively charged surface is sufficient to distinguish the small differences in peptide acidity introduced due to deamidation.

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However, not all PS C18 columns behaved equally: (i) Luna Omega PS-C18 providing 3 major peaks assigned to deamidated forms (Asp and IsoAsp) of the VVSVLTVLHQDWL**N**GK peptide, (ii) Biozen Peptide PS C18 providing moderate resolution of 4 peaks, which include the deamidated forms (Asp and IsoAsp) of the VVSVLTVLHQDWL**N**GK and a di-deamidated form of VVSVLTVL**HQ**DWL**N**GK peptide not detected earlier, and (iii) Kinetex PS C18 not being able to separate any of these. Hence, better separation of deamidated forms is achieved when using a positively charged surface chemistry combined with fully porous particles. Additionally, it is possible that the BioTi inert hardware contributed to higher recoveries of these acidic peptides, detecting an additional deamidated form when the Biozen Peptide PS C18 was used. This supports the importance of selecting both: (i) the appropriate column chemistry, as reflected by the lack of separation seen when using Polar C18 chemistry, and (ii) the most suitable particle morphology (core-shell vs. fully porous) for enhanced interactions.

Electron activation dissociation (EAD) fragmentation has the unique ability to generate signature fragments for Asp and IsoAsp, with IsoAsp having a mass shift of -57 Da. **Figures 19** through **22** show EAD results that confirm the identity of IsoAsp.

Figure 18. EIC (**Left**) and Zoomed EIC (**Right**) of native, deamidated (IsoAsp) and deamidated variants of peptide VVSVLTVLHQDWL**N**GK. (**A**) Chromatograms on a Luna Omega 3 µm PS C18, (**B**) Biozen 3 µm Peptide PS C18, (**C**) Kinetex 2.6 µm PS C18 and (**D**) Luna Omega 3 µm Polar C18 columns. Retention time in minutes.

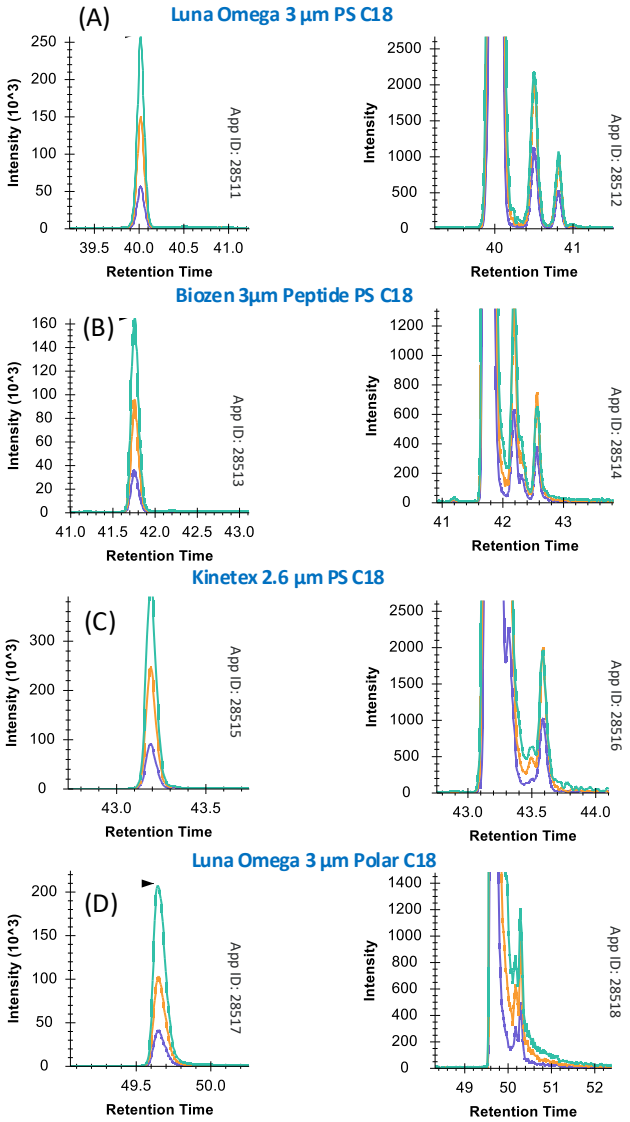


Figure 19. (**A**) EIC of native, deamidated (IsoAsp) and deamidated (Asp) variants of VVSVLTVLHQDWL**N**GK and (**B**) Zoomed in version on a Luna™ Omega 3 µm PS C18 using EAD MS/MS approach. Retention time (RT) in minutes

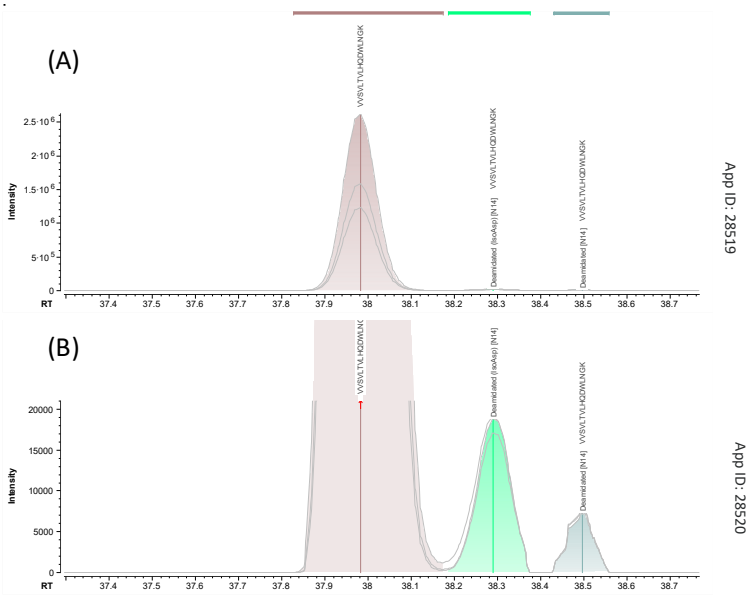


Figure 20. Peptide fragment spectra showing full sequence coverage for the deamidated (IsoAsp) version of VVSVLTVLHQDWL**N**GK on a Luna Omega 3 µm PS C18 using EAD MS/MS approach.

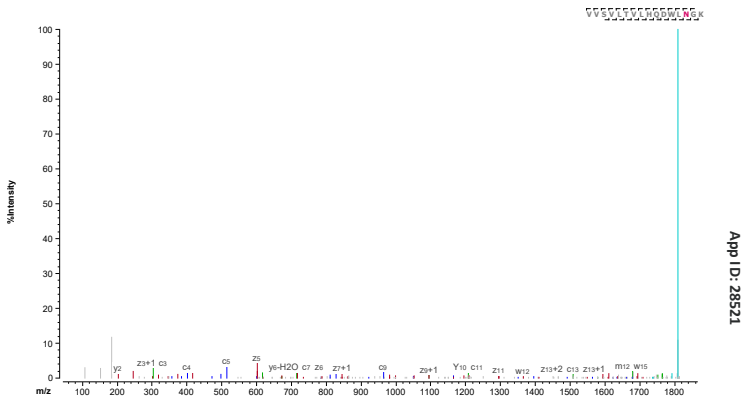
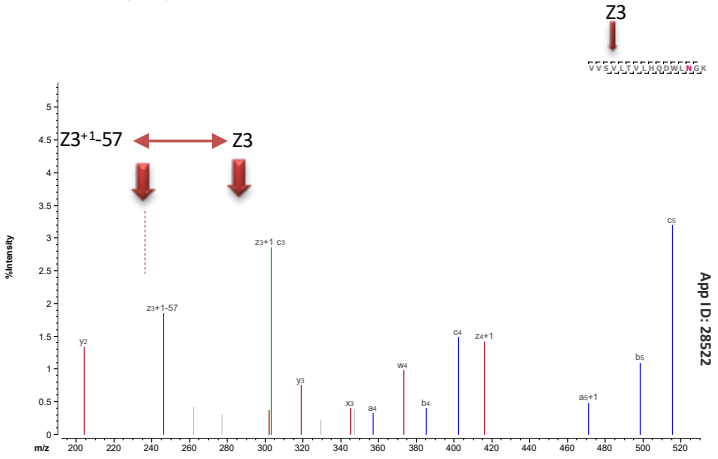


Figure 21. Zoom of peptide fragment spectra showing the $Z3^{+1}$-57 signature EAD fragment belonging to amidated (IsoAsp) of VVSVLTVLHQDWL**N**GK on a Luna Omega 3 µm PS C18.



Conclusions

The assessment of quality critical attributes of biotherapeutic proteins by peptide mapping must provide adequate resolution and recovery of a broad set of peptides. Selecting a column for peptide mapping is a challenge, not only due to the multitude of column manufacturers, but most importantly, due to the variety in degree of selectivity within the reversed phase chemistries. The diverse physicochemical properties of peptides, such as size, polarity and charge, make elution prediction difficult, frequently requiring multiple column screening processes to better determine the column that can provide the needed separation.

We have explored the effect of column attributes, such as peak capacity, retention, and selectivity, by systematically evaluating the peptide mapping performance of 13 reversed-phased columns belonging to three distinct column chemistry families. A major finding is the positive effect introduced using bioinert hardware (BioTi™), as these columns often provided the highest peak capacity and reproducibility when assessing a mAb digest. The three main chemistries to evaluate when working with peptide mixtures are clearly XB-C18, Polar C18 and PS C18. The XB-C18 chemistry showed a balanced performance for most peptide properties, whereas Polar C18 proved particularly useful when working with early eluting polar peptides, showing better retention and peak shape. The PS C18 chemistry provided outstanding results for acidic and basic peptides, with the additional feature of achieving good separation of deamidated peptide forms. These differences in selectivity can prove particularly useful when problematic peptides, such as peptides with low retention or poor peak shape, are needed for sequence coverage and CQA analyses.

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