High Resolution Glycopeptide Mapping of EPO Using an Agilent AdvanceBio Peptide Mapping Column

Application Note

BioPharma

Authors

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Abstract

The 2.7 µm Agilent AdvanceBio Peptide Mapping column is specifically designed to improve separations of peptide and peptide mapping applications. The column is based on superficially porous technology and was developed for fast and efficient separation of complex peptide mapping mixtures. This application note demonstrates peptide mapping performance with the AdvanceBio Peptide Mapping column for profiling a tryptic digest of recombinant human erythropoietin (rhEPO) protein as a model digest for glycopeptide profiling. Like many protein therapeutics, rhEPO exhibits a great deal of heterogeneity due to modifications that occur during manufacturing. The AdvanceBio Peptide Mapping column, with an optimized C18 coating technology, provides excellent peptide selectivity and resolution across a broad elution range to enhance separation of the glycopeptide fragments for fast, efficient, and sensitive mass spectrometry analysis.

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Introduction

Peptide mapping using reversed-phase HPLC of proteolytic peptides, combined with electrospray ionization mass spectrometry (ESI/MS), has become the method of choice for establishing the identity and efficacy of biotherapeutic products [1]. Peptide fragment analysis by liquid chromatography/mass spectrometry (LC/MS) is a crucial requirement for the release of these products and plays an important role to complete the protein characterization strategy. For example, the stability of a recombinant protein therapeutic requires long term monitoring throughout the shelflife of the product for important modifications, such as glycosylation. Although glycosylation can be analyzed on the intact protein, glycopeptide mapping is necessary to provide additional information of critical importance, such as sequence information, mass analyses, and identification of the glycosylation sites.

This application note used an rhEPO protein as a model glycoprotein to demonstrate high resolution peptide mapping and glycopeptide analysis by LC/MS using a 2.7 µm AdvanceBio Peptide Mapping column.

The column was specifically designed to improve separations for peptide mapping applications and provided increased resolution, higher analytical sensitivity, and greater selectivity needed for high efficiency peptide profiling, such as that required for rhEPO mapping and enhanced glycopeptide mining. Furthermore, the 2.7 µm superficially porous particles enabled the use of a longer column (2.1× 250 mm) at a higher flow rate delivering sub-2 µm type resolution within HPLC column pressures.

Materials and Methods

Recombinant human EPO was purchased from Creative Biolab, Shirley, NY. Triflouroacetic acid was purchased from Sigma-Adrich Corp., St. Louis, MO, and iso-propanol and acetonitrile were supplied from Honeywell-Burdick & Jackson, Muskegon, MI. High quality sequence-grade trypsin was obtained from the Stratagene division of Agilent Technologies.

Protease digestion was accomplished by adding trypsin protease to a solution including approximately 4.2 mg (2.1 mg/mL, 2 mL) EPO. The ratio of substrate and enzyme was 50:1 (w:w). The mixed solution was incubated at 37 °C for 12 hours. The digestion was quenched by storing the sample at –70 °C. After validation, 3.78 mg (2.1 mg/mL, 1.8 mL) of digested EPO was obtained.

An Agilent 1200 Infinity LC System was used, with auto injector (HiP-ALS), binary pump, thermostatted column compartment (TCC) and diode array detector (DAD), coupled to an Agilent 6224 TOF LC/MS. Data was processed using an Agilent MassHunter Qualitative Analysis software and an Agilent MassHunter BioConfirm.

TOF MS parameters

Spectra were recorded in positive ion and in centroid mode.

Gas temperature:	350 °C
Drying gas:	10 L/min
Nebulizer:	45 psi
V_{cap}:	3,500 V
Fragmentor:	170 V
Skimmer:	65 V
Octapole 1 RF:	750 V
MS:	4 Hz
Mass range:	100 to 7,000 *m/z*
Reference mass:	149.02332 922.009798
Acq. mode:	Extended dynamic range mode (2 GHz)

Chromatographic conditions

Column:	Agilent AdvanceBio Peptide Mapping, 2.1× 250 mm, 2.7 µm (p/n 651750-902)
Eluent:	A, H_2O + 0.1% formic acid (v/v); B, acetonitrile + 0.1% formic acid (v/v)
Injection volume:	5 µL (2 µg/µL)
Flow rate:	0.4 mL/min

Gradient:

Time (min)	% B
0	3
28	45
33	60
34	95

Temperature: 55 °C

Results and Discussion

Optimized rhEPO HPLC peptide mapping with an AdvanceBio Peptide Mapping column

Systematic method development was performed for mapping the rhEPO digest to determine the best resolved separation under water/ACN conditions. During these evaluations, 2.1 × 150 mm and 2.1 × 250 mm AdvanceBio Peptide Mapping columns were evaluated under various flow, temperature, and gradient profiles. Additionally, since peptide mapping separations work well under conditions that are favorable for ESI/MS, separations were evaluated with trifluoroacetic acid (TFA) and formic acid (FA) ion-pair additives to determine effects on retention and peak shape. The AdvanceBio Peptide Mapping column delivered excellent separation performance and compatibility with both TFA and FA ion-pair additives. The optimized separation displayed in Figure 1 provided the best separation profile for rhEPO mapping. The 2.1 × 250 mm AdvanceBio Peptide Mapping column and optimized chromatographic conditions with formic acid as the ion-pair additive enabled a high resolution separation of the rhEPO tryptic digest, providing very narrow peak widths, increased sensitivity, and unique selectivity across the gradient profile, making this separation highly amendable to ESI/MS analysis.

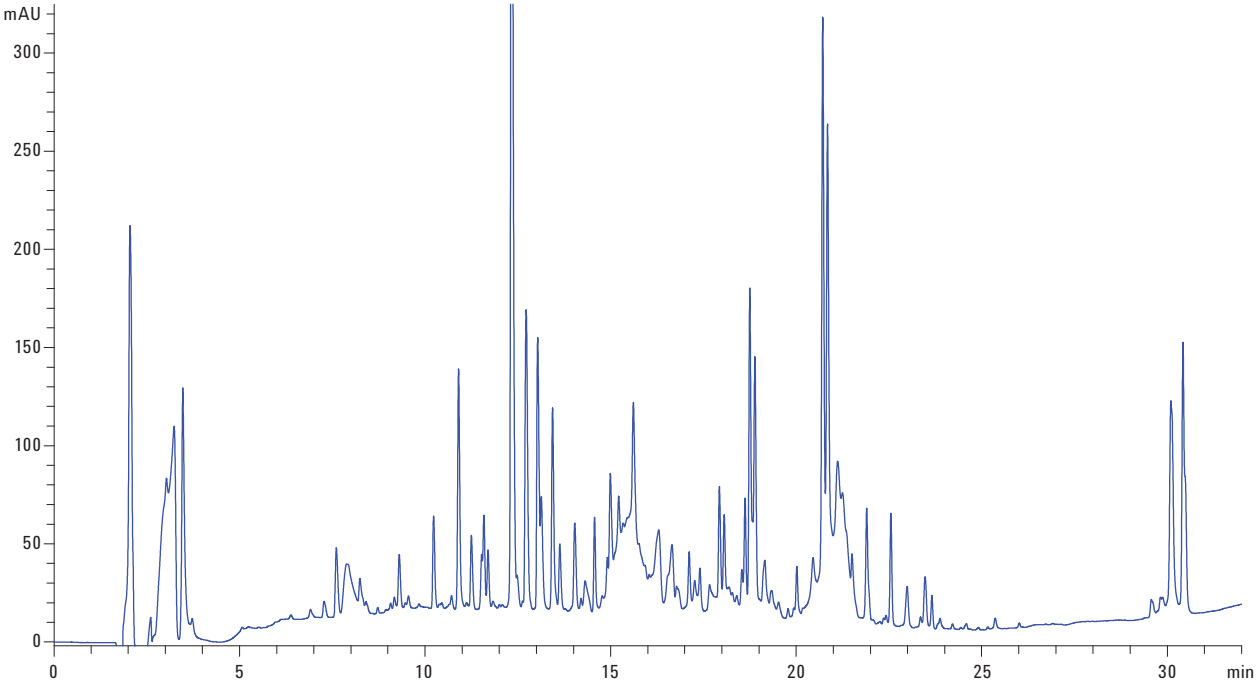

Figure 1. Reversed-phase rhEPO peptide map using a 2.1 × 250 mm, 2.7 µm Agilent AdvanceBio Peptide Mapping column (See Chromatographic conditions section).

Rapid rhEPO tryptic digest mapping by ESI-MS

Peptide maps often take 2 hours or longer using a 2.1 × 250 mm column to produce the needed resolution. Additionally, re-equilibration and run-to-run cycle times can add extended time to the completed analysis, significantly affecting laboratory production. While faster maps are desirable, it is critically important that resolution is not compromised. Figure 2 shows the total ion chromatogram (TIC) during a fast LC/MS analysis on a 2.1 × 250 mm column completed in less than 35 minutes. Excellent peak retention, peak shapes, and resolution across the gradient are all indicators of a robust method for LC/MS validation of the peptide mapping analysis.

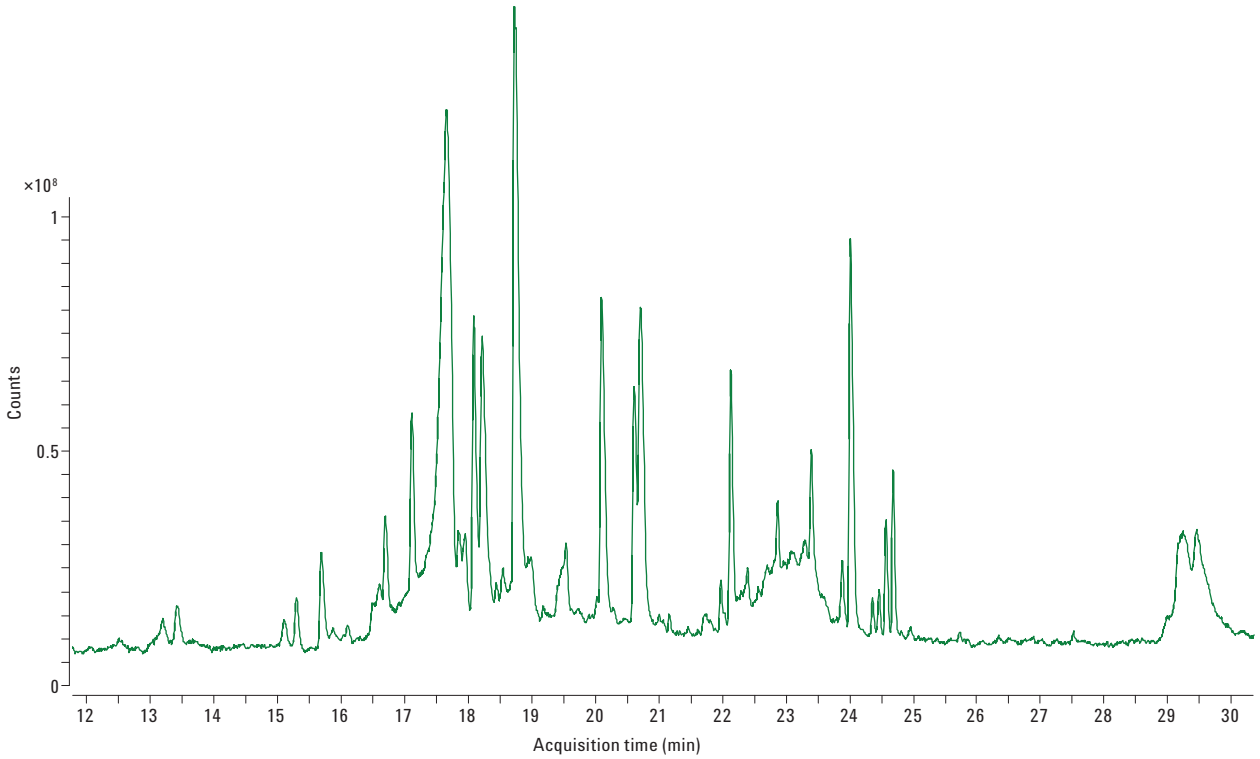

Figure 2. Total ion chromatogram of an LC/MS analysis on a 2.1 × 250 mm Agilent AdvancedBio Peptide Mapping column accomplished in under 35 minutes.

The faster analysis gained from the AdvancedBio Peptide Mapping column did not compromise the chromatographic map of rhEPO separation. Figure 3 shows the results from the Agilent MassHunter Molecular Feature Extractor (MFE). MFE is an algorithm that finds compounds from complex data and creates averaged MS spectra for each compound. These MFE compound results are then matched back to the rhEPO protein sequence. This 34 minute LC/MS method resulted in 100% sequence coverage of the rhEPO protein.

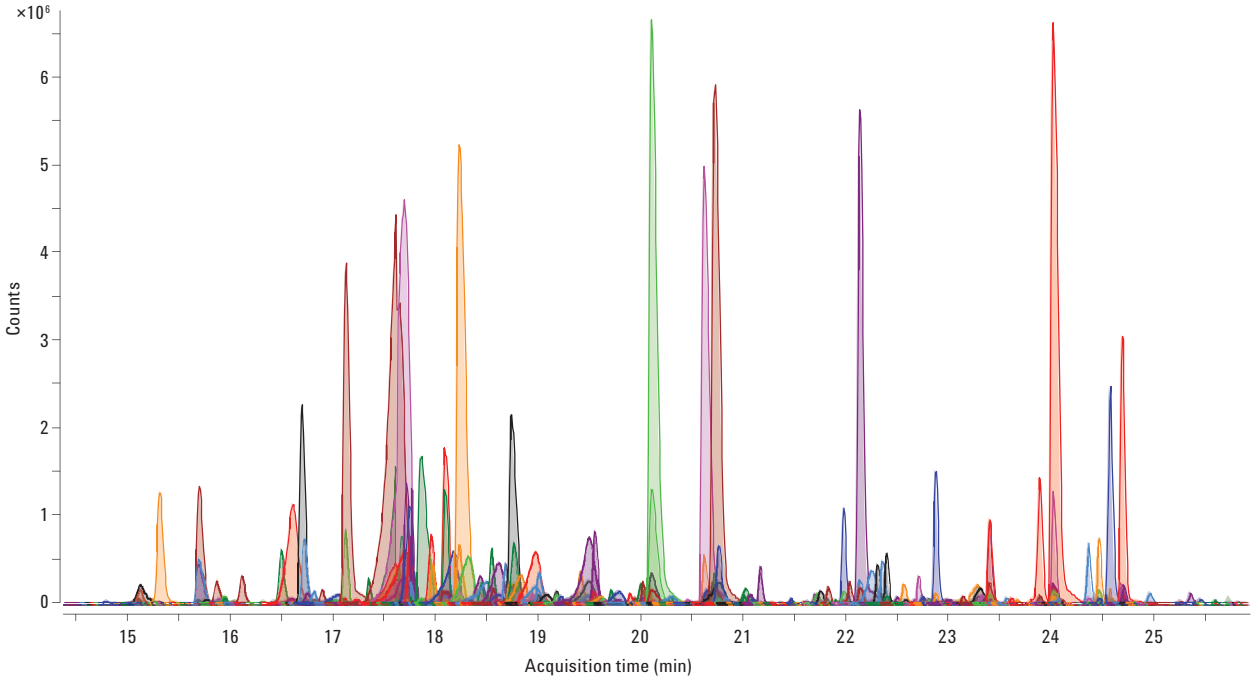

Figure 3. 100% rhEPO sequence coverage achieved using the 2.1 × 250 mm Agilent AdvanceBio Peptide Mapping column. Data generated using the MFE in Agilent MassHunter qualitative analysis software.

The rhEPO has a predicted molecular mass of 24,000 Dalton and apparent glycosylated molecular mass of 30,400 Dalton, and, thus, serves as an effective glycoprotein model to demonstrate glycopeptide profiling using the AdvanceBio Peptide Maping column. Figure 4 is the extracted peptide glycosylation profile from the total LC/MS analysis shown in Figure 3, while Table 1 shows the mass and glycosylated sequence information. The AdvanceBio Peptide Mapping column enabled the identity of 42 unique glycopeptides and demonstrated the performance advantage and utility of this column for highly complex peptide mapping, where post-translational modification information, such as glycosylation, is critical to the complete protein characterization.

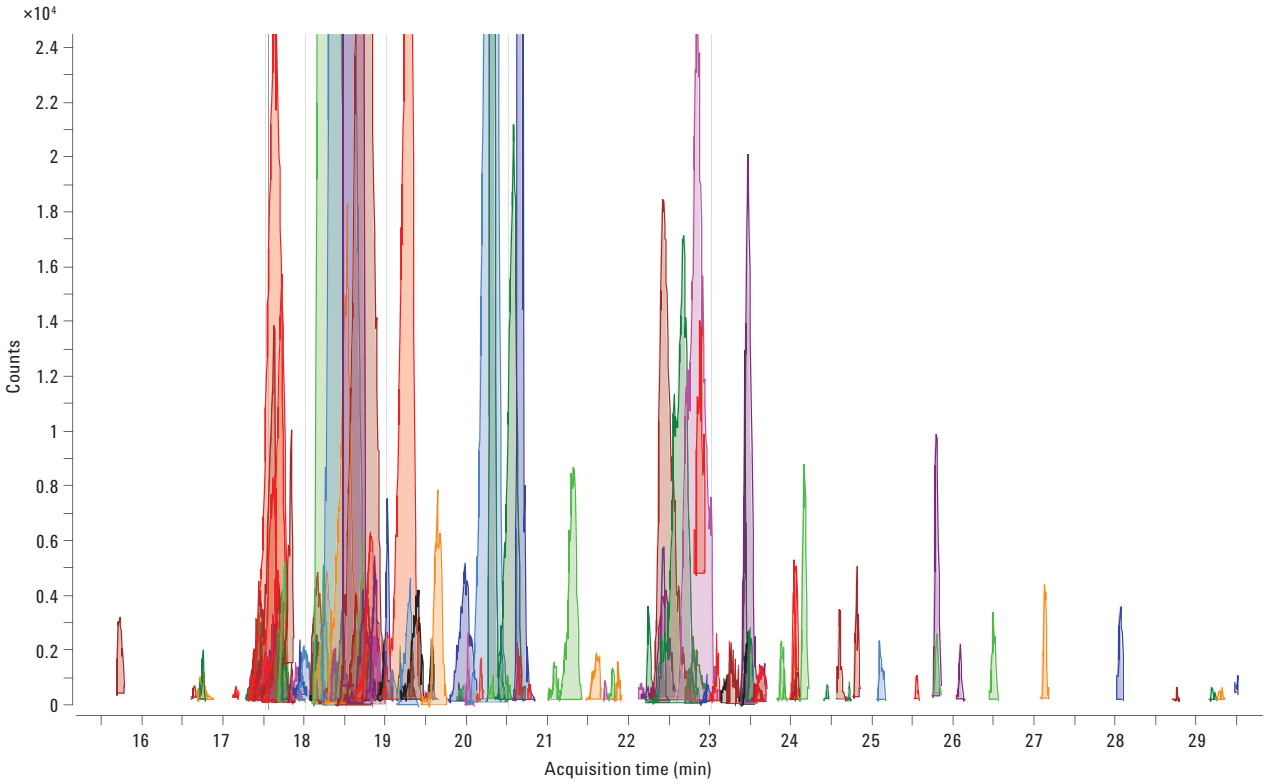

Figure 4. MFE extracted peptide glycosylation profile using the 2.1 × 250 mm Agilent AdvanceBio Peptide Mapping column. Data generated using Agilent MassHunter Molecular Feature Extractor (MFE) on an Agilent 6224 TOF LC/MS.

Table 1. Predicted N-linked glycan modifications and sequence for recombinant human EPO.

RT	Glyco-Peptide Accurate Mass	Pred glycan modification [seq. location]	Sequence
22.796	4272.681	1*G1F/G2F [A147]	VYSNFLR
24.569	4396.802	1*G1/G2 [A147]	LFRVYSNFLR
22.348	4110.643	1*G0F (NGA2F)/G2F A147	VYSNFLR
22.795	4218.745	1*G0 (NGA2)/G1F [A147]	LFRVYSNFLR
20.762	3669.49	1*G0 (NGA2)/G0F (NGA2F) [A47]	VNFYAWK
19.747	3798.577	1*3132 2A 0G [A147]	VYSNFLRGK
19.909	3613.416	1*3132 2A 0G [A147]	VYSNFLR
20.396	4039.697	1*3132 2A 0G [A147]	VYSNFLRGKLK
13.212	5075.323	1*3130 0A 0G [A83]	GQALLVNSSQPWEPLQLHVDKAVSGLR
22.404	3027.207	1*3111 1A 0G [A47]	VNFYAWK
26.057	2998.23	1*3111 1A 0G [A147]	VYSNFLR
23.922	2730.163	1*3100 0A 0G [A47]	VNFYAWKR
20.642	3668.512	1*3032 1A 1G or 3122 0A 2G [A147]	VYSNFLRGK
20.773	3668.505	1*3032 1A 1G or 3122 0A 2G [A147]	VYSNFLRGK
22.712	3737.536	1*3022 1A 1G [A147]	LFRVYSNFLR
19.456	2746.161	1*3010 0A 0G [A47]	VNFYAWKR
24.12	4456.815	1*2120 0A 0G (G2F) (NA2F) [A24]	EAENITTGCAEHCSLNENITVPDTK
16.278	2527.093	1*2100 0A 0G (G0F) (NGA2F) [A47]	VNFYAWKR
19.283	3147.255	1*2022 1A 1G [A47]	VNFYAWK
17.752	3243.417	1*2021 0A 1G [A47]	LFRVYSNFLR
26.01	3065.322	1*2011 1A 0G [A147]	LFRVYSNFLR
20.296	2665.073	1*2011 0A 1G [A147]	VYSNFLR
18.735	9045.706	1*2011 0A 1G 1*G1F/G2F[A24A36]	VLERYLLEAKEAENITTGCAEHCSLNENITVPDTK
17.767	8505.473	1*2011 0A 1G 1*3022 1A 1G [A24A36]	YLLEAKEAENITTGCAEHCSLNENITVPDTKVNFYAWK
17.979	7044.846	1*2010 0A 0G (G1)1*3030 0A 0G [A24A36]	EAENITTGCAEHCSLNENITVPDTKVNFYAWK
23.399	7653.184	1*2000 0A 0G (G0) (NGA2)1*3032 0A 2G [A24A36]	EAENITTGCAEHCSLNENITVPDTKVNFYAWKR
24.019	6396.629	1*2000 0A 0G (G0) (NGA2)1*3000 0A 0G [A24A36]	EAENITTGCAEHCSLNENITVPDTKVNFYAWK
16.927	2777.212	1*1111 1A 0G [A147]	VYSNFLRGK
18.433	2300.996	1*1110 0A 0G [A147]	VYSNFLR
23.401	2565.197	1*1100 0A 0G [A147]	VYSNFLRGKLK
17.586	2462	1*1011 0A 1G [A147]	VYSNFLR
23.885	3063.376	1*1011 0A 1G [A147]	LFRVYSNFLRGK
15.701	7365.966	1*1011 0A 1G 1*2022 2A 0G [A24A36]	EAENITTGCAEHCSLNENITVPDTKVNFYAWK
19.961	2340.001	1*1010 0A 0G [A47]	VNFYAWKR
20.285	2340.006	1*1010 0A 0G [A47]	VNFYAWKR
17.868	7570.105	1*1010 0A 0G 1*3132 2A 0G [A24A36]	EAENITTGCAEHCSLNENITVPDTKVNFYAWK
24.685	7518.254	1*1010 0A 0G 1*2040 0A 0G [A24A36]	YLLEAKEAENITTGCAEHCSLNENITVPDTKVNFYAWK

Table 1. Predicted N-linked glycan modifications and sequence for recombinant human EPO (continued).

RT	Glyco-Peptide Accurate Mass	Pred glycan modification [seq. location]	Sequence
19.896	1992.853	1*1000 0A 0G [A147]	VYSNFLR
22.134	7293.135	1*1000 0A 0G 1*3031 0A 1G [A24A36]	VLERYLLEAKEAENITTGCAEHCSLNENITVPDTK
29.128	7842.206	1*0100 0A 0G 1*G0F (NGA2F)/G1F [A24A36]	EAENITTGCAEHCSLNENITVPDTKVNFYAWKR
22.303	6912.043	1*0100 0A 0G1*3011 0A 1G [A24A36]	VLERYLLEAKEAENITTGCAEHCSLNENITVPDTK
17.671	6095.53	1*0100 0A 0G1*2010 0A 0G (G1) [A24A36]	EAENITTGCAEHCSLNENITVPDTKVNFYAWK

Conclusions

The Agilent 2.7 µm AdvanceBio Peptide Mapping column demonstrated excellent utility for fast and efficient LC/MS peptide mapping using a recombinant human erythropoietin protein digest. A 2.1 × 250 mm column dimension, and chromatographic conditions optimized for mass spectrometry analysis, provided high resolution separation of the tryptic rhEPO digest across the entire gradient profile demonstrating unique selectivity and retention features critical to generating a well-defined and resolved peptide map. The AdvanceBio Peptide Mapping column enabled 100% rhEPO sequence coverage by ESI/MS and provided 42 glycopeptide matches, making this column an excellent choice for highly complex peptide mapping applications.

Reference

1. L. J. Campbell, J. Y. Le Blanc. *Bioanalysis*, 3, 645 (2011).

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