



Fast and Efficient Peptide Mapping of a Monoclonal Antibody (mAb): UHPLC Performance with Superficially Porous Particles

Application Note

Biotherapeutics and Biosimilars

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Introduction

Peptide mapping by reversed-phase (RP) chromatography is the mainstay technique in biopharmaceutical analysis, delivering comprehensive characterization of biopharmaceutical products. When interfaced with a mass spectrometer (MS), it can deliver the identification of proteins and their variants, determine post-translational modifications (PTMs) and locations, and confirm protein sequences. However, peptide mapping represents a significant chromatographic challenge due to the inherent complexity of protein digests. As a result, many organizations struggle with developing robust and reliable peptide maps. In general, peptide maps have suffered from low sensitivity, poor peak shapes, and very long separation times to achieve the desired resolution.

More recently, Ultra High Performance Liquid Chromatography (UHPLC) has been employed to overcome these challenges, demonstrating superior resolution, higher sensitivity, and much shorter analysis times compared to traditional HPLC. UHPLC technology can provide the basis for a more detailed characterization of protein biopharmaceuticals and takes advantage of faster flow rates, smaller particles, and shorter column lengths to achieve high separation performance during much faster run times. However, the pressure requirement becomes increasingly high for these analyses and prevents operation on traditional 400 and 600 bar HPLC instruments, thus severely limiting its broader use.



Agilent Technologies

To address this limitation, Agilent has introduced a 2.7 μm AdvanceBio Peptide Mapping column to fill a critical gap in biotherapeutics characterizations, for generating both rapid and highly efficient peptide maps at traditional LC-system pressures. Using superficially porous chromatographic media, AdvanceBio Peptide Mapping columns achieve substantial improvements in peptide mapping during very fast run times and low system pressures, while still maintaining high peak-performance efficiency. In this work, an AdvanceBio Peptide Mapping column was used for the LC/MS peptide mapping analysis of a monoclonal antibody (mAb) tryptic digest with reduced analysis times. Additionally, the AdvanceBio column and methodology was compared to a sub-2 μm non-Agilent UHPLC peptide mapping column for performance comparisons.

Materials and Methods

Sample preparation

Fifty microliters mAb IgG1 (30 mg/mL) were first mixed with 75 μL 100 mM ammonium bicarbonate (pH 8). Seventy-five microliters of trifluoroethanol and 3 μL 200 mM dithiothreitol (DTT) were added to the protein sample and heated at 60 $^{\circ}\text{C}$ for 1 h to denature and reduce the protein. After the protein had cooled to room temperature, 12 μL 200 mM iodoacetamide (IAM) was added to the sample. The sample was kept at room temperature in the dark for 1 h. Subsequently, 3 μL DTT was added in the sample to react with the excess IAM for 1 h. The sample was diluted with 900 μL water and 300 μL 100 mM ammonium bicarbonate. Trypsin solution (75 μL) was added to the sample and incubated at 37 $^{\circ}\text{C}$ for 20 h. After overnight incubation, 3 μL of neat formic acid was added to quench the digest.

Conditions

Columns:	AdvanceBio Peptide Mapping 2.1 \times 100 mm (p/n 655750-902), AdvanceBio Peptide Mapping, 2.1 \times 150 mm (p/n 653750-902), non-Agilent UHPLC column, 2.1 \times 100 mm
Eluent:	A: H ₂ O + 0.1% FA (v/v) B: 90% ACN + 0.1% FA (v/v)
Injection volume:	15 μL
Flow rates:	various
Temperature:	40 $^{\circ}\text{C}$
Detection:	UV, 215/220 nm
Instrumentation:	Agilent 1290 Infinity LC System and an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight (Q-TOF)

Q-TOF MS parameters

Ion mode:	Positive
Source:	Agilent Dual Jet Stream
Drying gas temperature:	250 $^{\circ}\text{C}$
Drying gas flow:	10 L/min
Sheath gas temperature:	250 $^{\circ}\text{C}$
Sheath gas flow:	12 L/min
Nebulizer:	35 psi
Capillary voltage:	3,500 V
Fragmentor:	200 V
Skimmer:	65 V
Oct 1 RF:	750 V
Nozzle:	0 V
MS range (<i>m/z</i>):	100 to 1600
MS/MS range (<i>m/z</i>):	100 to 1600
MS scan rate (spectra/second):	8
MS/MS scan rate (spectra/second):	3

LC/MS results were analyzed using Agilent MassHunter Qualitative Analysis Software B.06 and Agilent MassHunter BioConfirm Software B.06.

Results and Discussion

Fast peptide mapping optimization

Optimization of peptide maps to resolve and analyze potentially hundreds of peaks is a significant task. Performing these optimizations with high resolution during shortened analysis times can be even more challenging and time consuming. Employing long gradients of mAb tryptic peptide maps to achieve the desired resolution can take 120 minutes or longer, especially for large proteins like mAbs. While faster maps are highly desirable, resolution must not be compromised, while end test results need to provide the same level of information found during an extended run.

To demonstrate the AdvanceBio Peptide Mapping column's flexibility for increasing analysis speed without sacrificing separation performance, two UV separations are compared in Figure 1. In this comparison, a mAb tryptic peptide map was fully optimized at 75 minutes on a 2.1×150 mm AdvanceBio Peptide Mapping column (top chromatogram) with an

efficient baseline-resolved peptide map across the entire gradient profile. The 75 minute separation is an excellent example of the column's ability to enable faster separation performance for a 150 mm length column (traditional column length), compared to conventional peptide mapping times at this dimension. However, it also provides a better example for comparing the column's flexibility for further increasing analysis speed without sacrificing separation performance when moving to a shorter gradient.

In the bottom 14 minute chromatogram in Figure 1, column flow rate was increased from 0.2 to 0.6 mL/min while column length was decreased from 150 to 100 mm. Volumetric flow during this change was carefully maintained, keeping the gradient change per column volume equal to ensure similar selectivity. The 14 minute peptide map details excellent separation performance, maintaining the baseline resolution and sensitivity while keeping resolving power unchanged. The 14 minute separation demonstrates UHPLC- type speed and performance, yet keeps this rapid analysis attainable on 600 bar HPLC instrumentation.

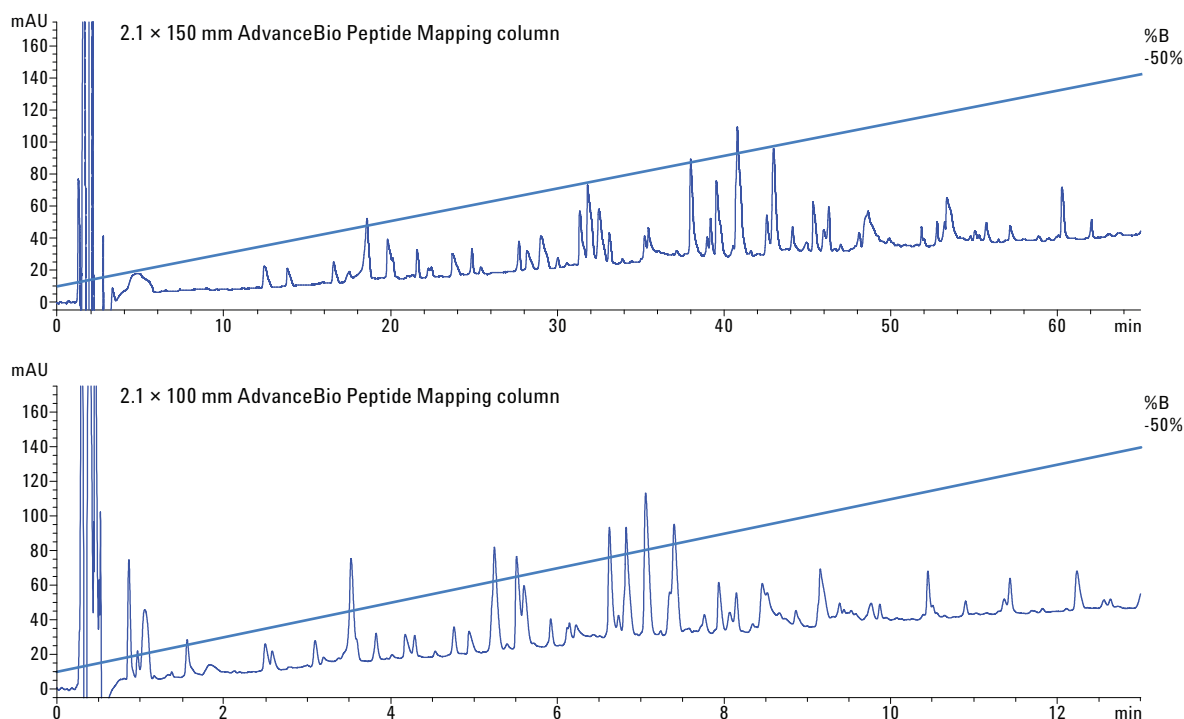


Figure 1. AdvanceBio Peptide Mapping column optimization for achieving a faster peptide mapping analysis. Gradient 10-40% B, DAD: 215 nm, 40 °C. Top panel, 75 minute separation on a 2.1×150 mm column generated 59 peptide peaks (flow rate 0.2 mL/min, 211 bar). Bottom panel, optimized 14 minute separation on a 2.1×100 mm column generated 57 peptide peaks (flow rate 0.6 mL/min, 433 bar).

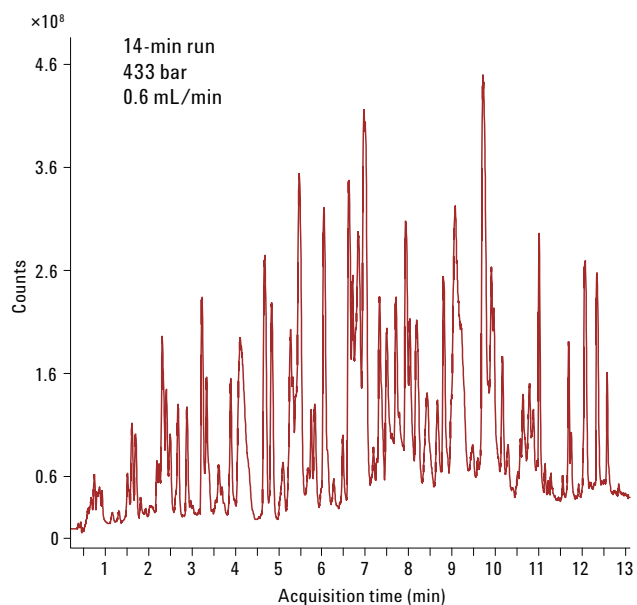
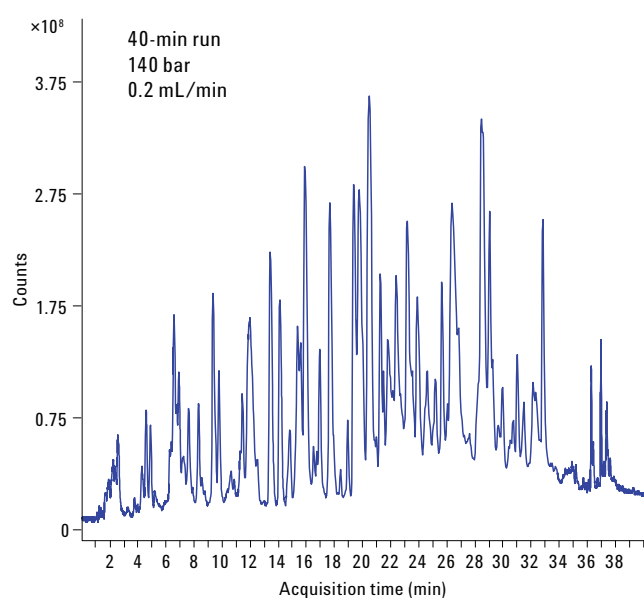
LC/MS peptide mapping with an AdvanceBio Peptide Mapping column: steep versus long gradient

In a typical biopharmaceutical peptide mapping workflow, reversed-phase LC is combined with mass spectrometry detection. Compared with the more traditional approach of UV only detection, RP LC/MS significantly enhances the information content available from the peptide-mapping experiment. Measurement by RP LC/MS can differentiate coeluting peptides, locate and identify peptide modifications, and determine the sequence coverage. High sequence coverage is an indicator that optimal separation of the tryptic digest has been achieved.

During RP LC/MS, long gradients are typically used to enable higher resolution and ensure modified peptides (referred to as post translational modifications or PTMs) are detectable from their unmodified (native) forms as well as other close eluting

peptides within the overall digest profile. To reduce run times steeper gradients are employed, however, this is typically at the cost of lost resolution and overall mapping quality. It is, therefore, important to not sacrifice separation quality or compromise mass spectral information when run times are reduced.

Figure 2 compares peptide mapping total ion chromatograms (TIC) from an AdvancedBio Peptide Mapping column during 40-min and 14-min runs. In this comparison, column dimensions remained constant, and the gradient slope was adjusted to keep gradient change/column volume between the columns equal, ensuring that the chromatographic selectivity remained similar. The TIC comparisons showed that resolution, selectivity, and separation performance had not been compromised during the reduced run time of 14 min, while the increase in pressure at the higher flow rate did not require > 600 bar UHPLC instrumentation.



Gradient, 40 minute		Gradient, 14 minute	
Time (min)	%B	Time (min)	%B
0	3	0	3
35	35	10	35
37	90	12	90
40	90	14	90

Figure 2. Comparison of Agilent AdvanceBio Peptide Mapping column total ion chromatograms (TICs), highlighting peptide mapping performance between 40-min and 14-min runs for an mAb tryptic digest. Left panel, TIC collected during 40-min analysis (flow rate 0.2 mL/min, 140 bar). Right panel, TIC collected during 14-min analysis. (flow rate 0.6 mL/min, 433 bar).

Additionally, sequence coverage between the 40-min and 14-min run was also evaluated using the Molecular Feature Extractor (MFE) in the MassHunter Qualitative Analysis Software. MFE is an algorithm that finds and extracts a list of compounds from complex separation data, such as peptide maps. The list of compounds is then matched back to the mAb protein sequence and provides the sequence coverage. Figure 3 displays the extracted compound chromatograms (ECCs) for the matched peptides and the sequence coverage.

All matched peptides were acquired with at least one MS/MS spectrum for confirmation. The 40-min and the 14-min runs remain relatively unchanged in coverage. The 14-min LC/MS run resulted in 99.63% sequence coverage of mAb while the 40-min analysis resulted in 99.84%. This comparison provides further confidence that peptide characterization information is not sacrificed when peptide mapping analyses times are shortened on the AdvanceBio Peptide Mapping column.

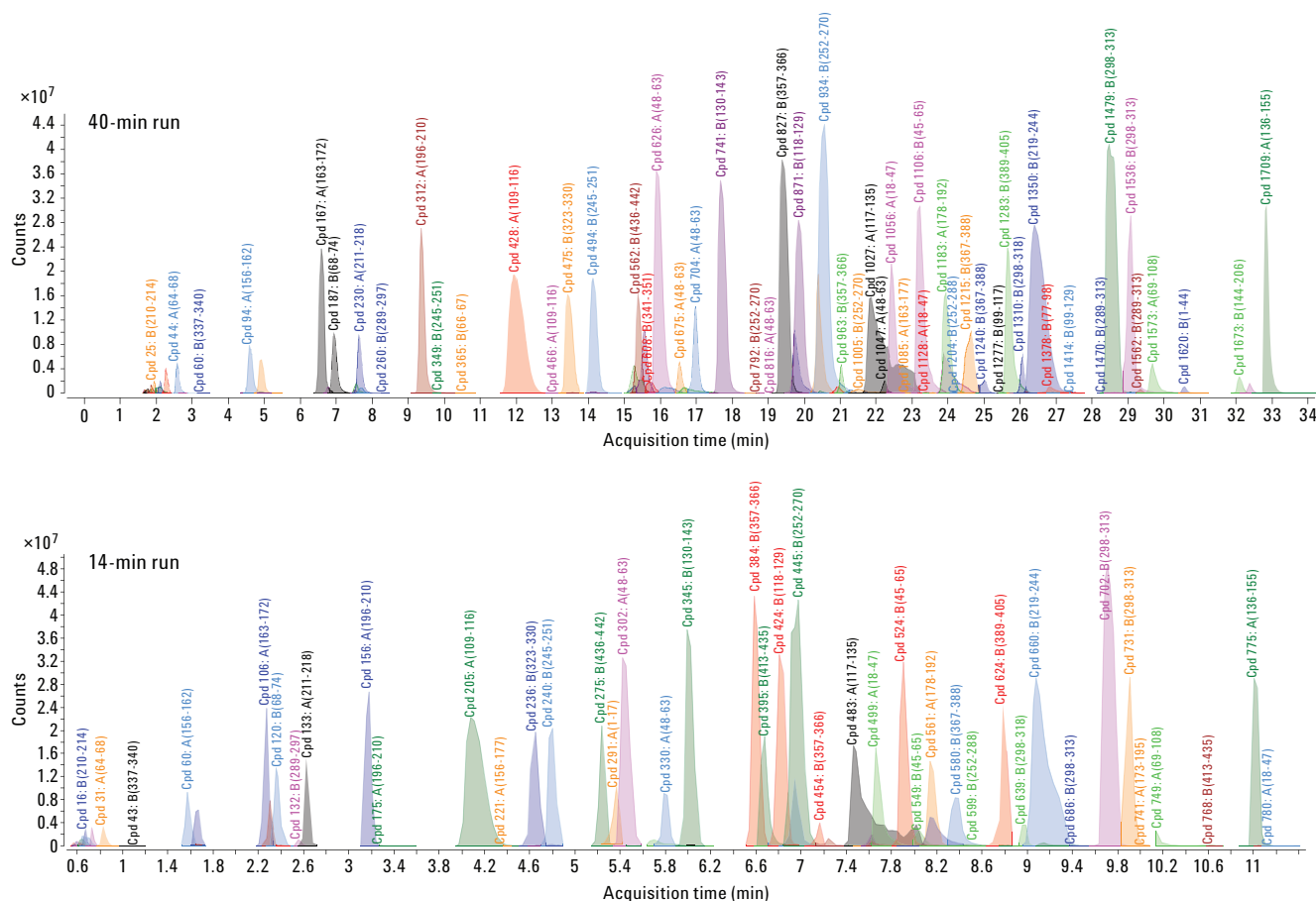


Figure 3. Agilent AdvanceBio Peptide Mapping column extracted compound chromatograms (ECC) to compare mAb tryptic digest results. The top panel shows an ECC for peptides identified from the 40-min run, resulting in 99.84% sequence coverage of mAb. The bottom panel shows an ECC for peptides identified from the 14-min run, resulting in 99.63% sequence coverage of mAb.

Rapid PTM profiling

Deamidation can cause structural and functional changes and is an important post translational modification to monitor during mAb discovery, development, and manufacturing. Figure 4 displays an example of how peptide deamidation was monitored and conserved during a fast run time of 14 minutes, in comparison to a longer run time of 40 minutes. Heavy chain peptide 357-366, which contains Asn 357, was identified in both peptide mapping separations. In the top and middle ECC figures, the native peptide peak was fully separated from its two deamidated forms, displaying as a total of three separate peaks in the overlaid ECCs. In comparison to the 40-min run (top), the native and deamidated species in the 14-min run (bottom) were still well resolved and readily identified by the QTOF analysis.

In the bottom figure, MS/MS of the native peptide (precursor at $m/z = 581.32$) and two deamidated forms (precursor at $m/z = 581.81$) are shown. In this spectra, the y and b-series fragments are the predominant ions, and all three peptides show the same y4-y8 ions, however the b2 and b3 ions (circled) are 0.98 Da higher for peak 2 and 3 thus validating the modified forms from the native species.

The automatic compound extraction (in MFE) also adds all the ion intensities (isotopes, charge states, adducts, and so forth) belonging to the peptides. These are listed under the Volume tab in peptide Table 1. The comparative percentage of modified peptides can then be easily derived from the two data sets in Table 1. In summary, the rapid 14 minute peptide map generated from the AdvanceBio Peptide Mapping column did not compromise PTM (deamidation) information or the sequence coverage in comparison to the longer run. Using a steeper gradient at increased flow, the column maintained excellent separation performance and delivered a high degree of confidence in a complete peptide mapping characterization.

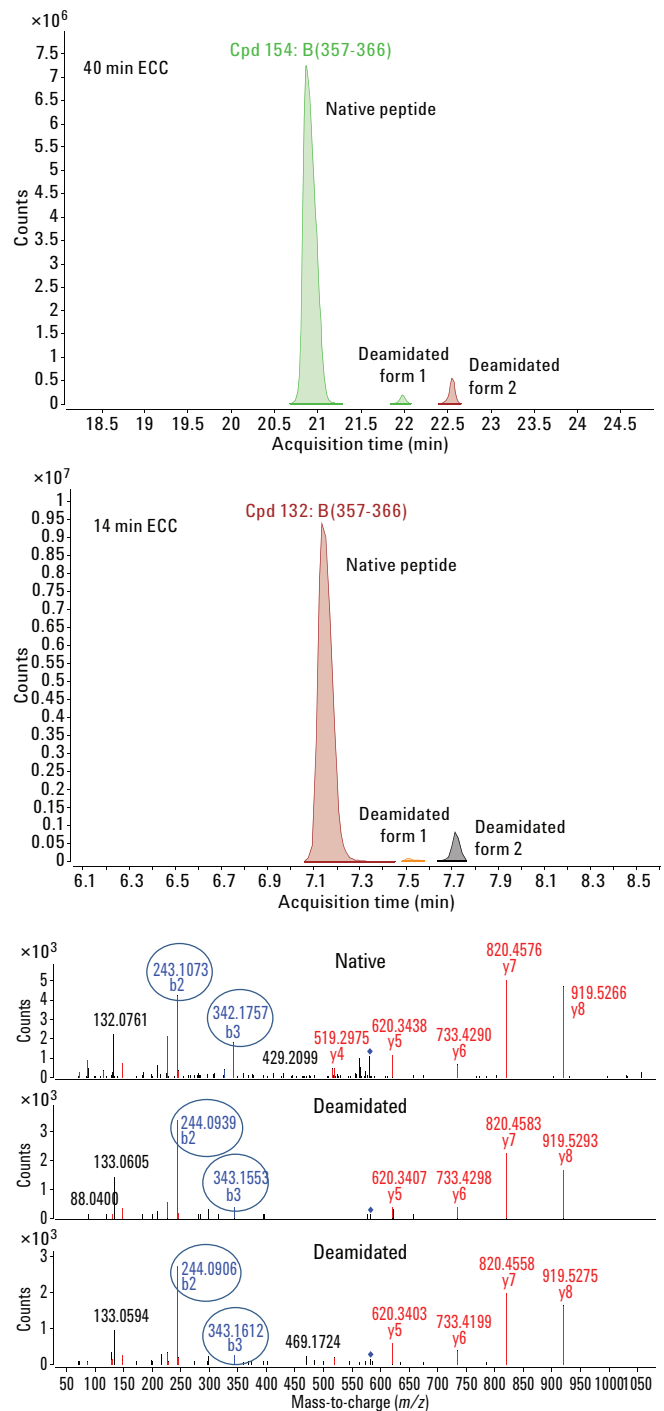


Figure 4. Overlaid extracted compound chromatograms (ECC) and MS/MS spectra (bottom) of native peptide and its deamidated forms. Upper panel displays results from the 40-min analysis. Middle panel displays results from the 14-min analysis. Bottom panel provides MS/MS spectra of the native and two deamidated forms shown above.

Table 1. Extracted compound list showing the native peptide HC (357-366) and two deamidated forms with their sequence, modification, retention time, mass and volume for the 40-min analysis (top) compared to the 14-min analysis (bottom).

40-min run

Show/Hide	File	Seq Loc	Sequence	Pred Mods	RT	Tgt Seq Mass	Vol	Score (Bio)
<input checked="" type="checkbox"/>	igg036-Agilent long run-peptides in -20.d	B(357-366)	NQVSLTCLVK		20.891	1160.6223	73748928	81.19
<input checked="" type="checkbox"/>	igg036-Agilent long run-peptides in -20.d	B(357-366)	NQVSLTCLVK	1*Deamidation(+0.984016)B357	21.98	1161.6064	1009838	59.65
<input checked="" type="checkbox"/>	igg036-Agilent long run-peptides in -20.d	B(357-366)	NQVSLTCLVK	1*Deamidation(+0.984016)B357	22.554	1161.6064	2747294	54.14

14-min run

Show/Hide	File	Seq Loc	Sequence	Pred Mods	RT	Tgt Seq Mass	Vol	Score (Bio)
<input checked="" type="checkbox"/>	igg035-Agilent short run-peptides in -20.d	B(357-366)	NQVSLTCLVK		7.145	1160.6223	41819052	73.17
<input checked="" type="checkbox"/>	igg035-Agilent short run-peptides in -20.d	B(357-366)	NQVSLTCLVK	1*Deamidation(+0.984016)B357	7.515	1161.6064	276957	70.69
<input checked="" type="checkbox"/>	igg035-Agilent short run-peptides in -20.d	B(357-366)	NQVSLTCLVK	1*Deamidation(+0.984016)B357	7.717	1161.6064	2147009	71.81

**Peptide mapping with steep gradients:
HPLC (AdvanceBio Peptide column) versus
UHPLC (non-Agilent UHPLC peptide column)**

With the advancement of UHPLC peptide mapping, separation performance has been significantly improved and analysis time shortened. With sub-2 μm particle technology, peptide resolving power can now be vastly improved during shortened runtimes; a consequence of reduced diffusional distance in small particles. This higher resolution and increased sensitivity becomes particularly important in peptide mapping, especially for the detection of modified peptides. In direct comparison to sub-2 μm column performance, 2.7 μm superficially porous particles with a thin porous outer shell and solid inner core can offer exceptional resolving power, which also results in fewer backpressure constraints as column flow rates are increased. This pressure and speed advantage can obviate the requirement for > 600 bar UHPLC instrumentation to perform these fast and highly efficient peptide mapping analyses.

The UV chromatographic comparison for an IgG tryptic digest in Figure 5 highlights the AdvanceBio Peptide Mapping column's speed and resolving power in direct comparison to a top industry performing UHPLC peptide mapping column. In the top chromatogram, the AdvanceBio Peptide Mapping column was optimized for a rapid and highly efficient analysis using 2.1 \times 100 mm dimensions. In this separation, the peptide peaks were well resolved across the entire gradient profile, delivering 56 peaks at a pressure of 433 bar. In the bottom chromatogram, a 2.1 \times 100 mm UHPLC peptide-mapping column was compared under the same chromatographic conditions and column dimensions. However, the critical aspect of the peptide-mapping results is highlighted by the low pressure requirement from the AdvanceBio Peptide Mapping column to achieve the same separation results. The UHPLC peptide column resulted in 700 bar backpressure and required the use of UHPLC instrumentation, thus limiting its broader application.

The ability to quickly, routinely and comprehensively run LC/MS analyses for mAb mapping helps to accelerate all facets of the therapeutic development process. However, these separations are typically performed on high pressure LCs to take full advantage of maximum efficiency and time savings. Superficially porous separations can offer the flexibility to operate on either UHPLC/MS or HPLC/MS instrumentation coupled to MS, to deliver rapid and high efficiency separations. To contrast the LC/MS peptide mapping performance, and pressure requirements between sub-2 μm UHPLC and superficially porous HPLC peptide-mapping columns, a 2.1 \times 100 mm AdvanceBio Peptide Mapping column was directly compared to a non-Agilent UHPLC peptide mapping column of the same dimension. Figure 6 displays the RP LC/MS TIC and ECC performance results between these two columns. Again all the identified

peptides were acquired with at least one MS/MS spectrum as confirmation.

In the TIC and ECC comparisons, the AdvanceBio Peptide Mapping LC/MS analysis delivered excellent separation and sequence coverage in comparison to the non-Agilent UHPLC peptide-mapping column. Sequence coverage for the AdvanceBio peptide Mapping column was 99.63%, while the UHPLC coverage was comparable at 99.0%. Both columns delivered identity for 76 total tryptic peptides. However, the UHPLC analysis resulted in 700 bar backpressure, while the AdvanceBio separation operated 500 bar. The ability to obtain UHPLC like results on the AdvanceBio Peptide Mapping column can make this column more attractive for obtaining rapid and highly efficient peptide maps with the flexibility of using traditional HPLC instrumentation.

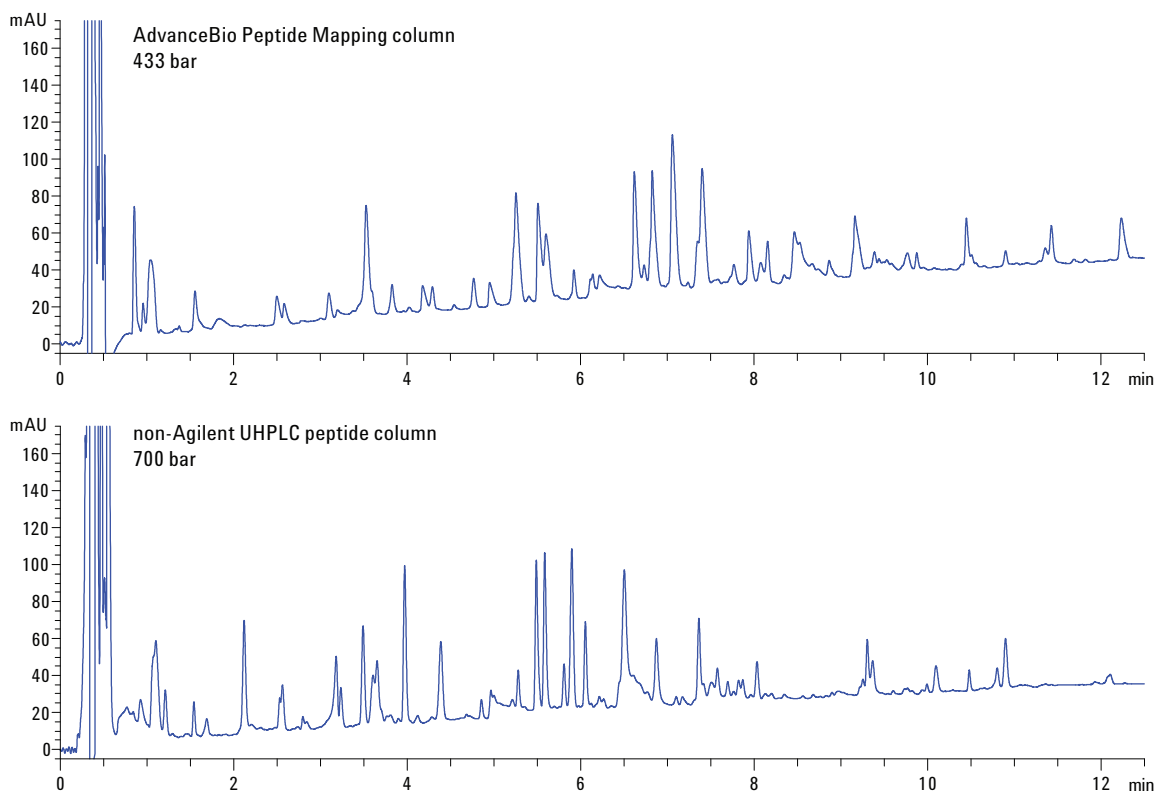


Figure 5. 2.1 \times 100 mm Agilent and non-Agilent UHPLC peptide maps of an mAb tryptic digest. Gradient: 10-40% B at 0.6 mL/min, DAD: 215 nm, temperature: 40 $^{\circ}\text{C}$, flow: 0.6 mL/min. Top, HPLC separation with an Agilent AdvanceBio Peptide Mapping column generated 56 peptide peaks at 433 bar. Bottom, UHPLC separation with a non-Agilent UHPLC peptide column generated 52 peptide fragment peaks at 700 bar.

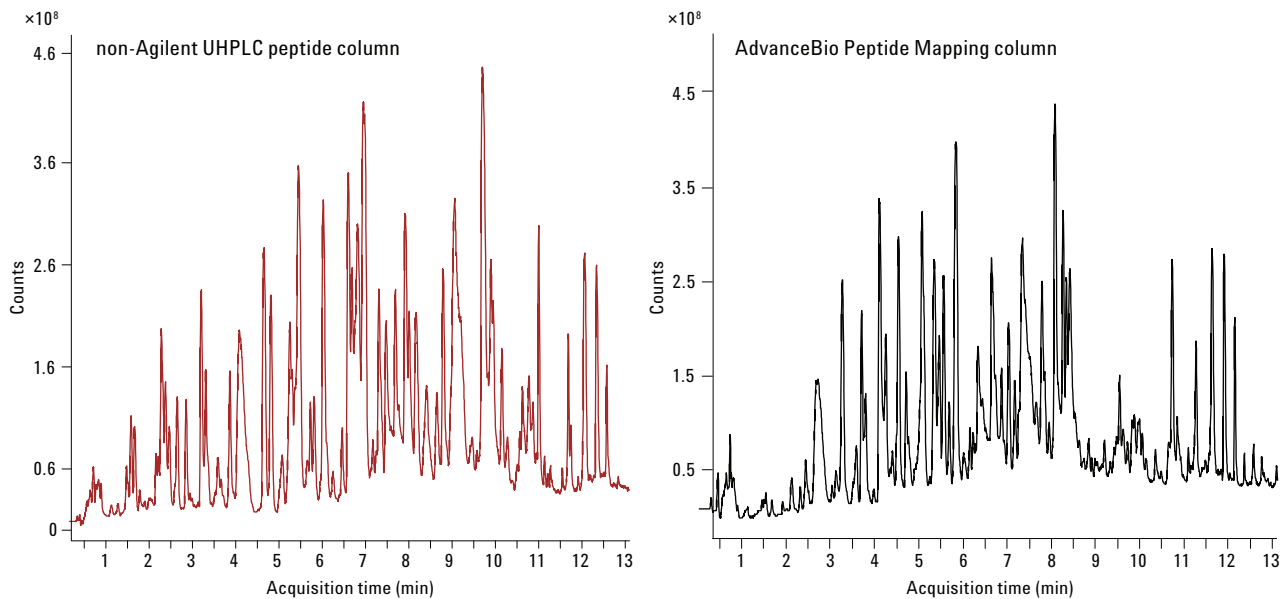


Figure 6A. Total ion chromatograms (TIC) for a mAb tryptic digest performed on a 2.1 × 100 mm Agilent AdvanceBio Peptide Mapping column, 433 bar (left panel) and 2.1 × 100 mm non-Agilent UHPLC peptide-mapping column, 700 bar (right panel). Chromatographic conditions same as Figure 5 conditions.

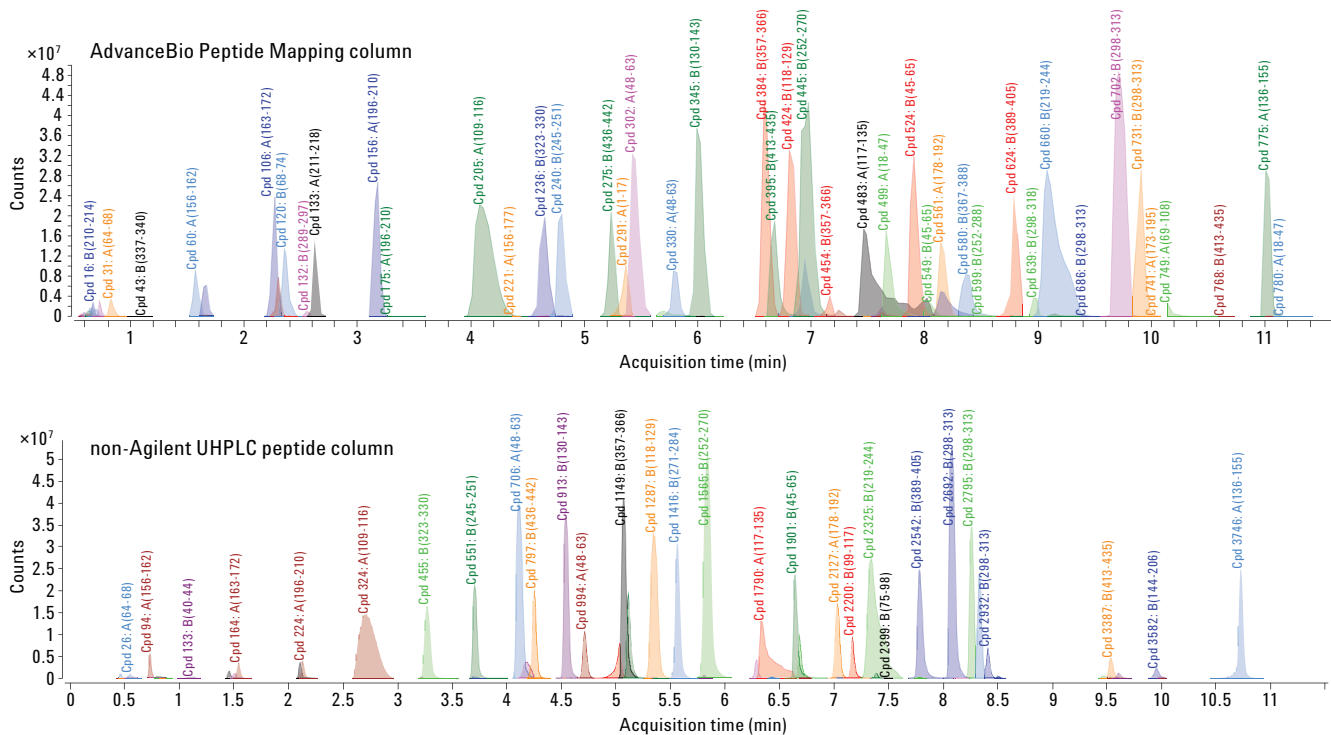


Figure 6B. Extracted compound chromatograms (ECC) for a mAb tryptic digest. Top panel, 2.1 × 100 mm Agilent AdvanceBio Peptide Mapping column, 433 bar, yielded 99.63% sequence coverage and 76 tryptic peptides identified. Bottom panel, non-Agilent UHPLC peptide-mapping column, 700 bar, yielded 99.0% sequence coverage and 76 tryptic peptides identified.

Conclusions

The AdvanceBio Peptide Mapping column greatly reduced the analysis time for RP LC/MS peptide mapping, delivering high resolution separations during fast runtimes. In combination with optimized gradient conditions, this application provides examples of highly efficient peptide mapping analysis for a monoclonal antibody tryptic digest in 14 minutes with low LC operating pressures (< 450 bar). In contrast to a non-Agilent UHPLC peptide-mapping column and fast analysis conditions, the AdvanceBio Peptide Mapping column generated rapid and well resolved peptide peaks across the entire gradient profile and resulted in high sequence coverage of mAb. Most importantly, the AdvanceBio Peptide Mapping separations were generated at low system pressures, highlighting the flexibility to run these separations on HPLC or UHPLC instrumentation.

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