Application Note

Biopharma

Agilent

Trusted Answers

Advancing Chromatographic Performance in Peptide Mapping

Using the Agilent Altura ZORBAX Eclipse Plus C18 inert column

Authors

Jordy Hsiao, Mahsan Miladi, Li Gu, Stephen Sciuto, Guannan Li, and Anne Blackwell

Agilent Technologies, Inc.

Abstract

This study highlights the performance benefits of the Agilent Altura ZORBAX Eclipse Plus C18 inert column for peptide mapping. Compared to stainless-steel hardware, the Altura column improves peak shape, reproducibility, and resolution, especially for hydrophobic and modified peptides. In LC/MS analysis of synthetic mAb peptides and trypsin-digested Herceptin, the inert column enabled confident identification of post-translational modifications, with improved peak shapes and resolution for deamidated peptides. Enhanced separation of deamidated variants demonstrates the column's value in biopharmaceutical workflows requiring high sensitivity and precision.

Introduction

Peptide mapping is a critical technique in biopharmaceutical development, used to confirm protein sequences and identify post-translational modifications (PTMs). However, interactions between analytes and metal surfaces within liquid chromatography (LC) hardware can compromise sensitivity and resolution, particularly for charged or phosphorylated peptides. Recent studies have underscored the importance of inert flow paths and column hardware in mitigating these challenges.[1,2]

In a previous study, a peptide mapping method using the AdvanceBio Peptide Mapping column was optimized to reduce run time from 2.5 hours to 1 hour by leveraging the sub-2 µm particles of the ZORBAX RRHD Eclipse Plus C18 column and a UHPLC system.[3] Despite the significant reduction in run time, comparable peptide elution patterns were observed between the AdvanceBio Peptide Mapping column and the ZORBAX RRHD Eclipse Plus C18 column, ensuring method transferability and consistent analytical results across different column technologies. This improvement supports higher sample throughput and reduces solvent consumption without compromising performance.

To further enhance performance, Agilent Altura ZORBAX Eclipse Plus C18 inert columns incorporate Ultra Inert technology, designed to minimize metal-analyte interactions while maintaining the mechanical strength of stainless steel (SS). In this study, we compare the performance of Altura inert columns with conventional SS columns using the Agilent 1290 Infinity II Bio LC and Agilent 6545XT AdvanceBio LC/Q-TOF systems for LC/MS/MS analysis of synthetic peptides and digested monoclonal antibody (mAb) samples.

Experimental

Sample preparation

Herceptin was digested with trypsin following a previously described protocol[4], with modifications. Specifically, digestion was performed with trypsin using a protein-to-enzyme ratio of 50:1. In the previous protocol, trypsin/LysC was used at a protein-to-enzyme ratio of 25:1 with no sample cleanup step.

Prior to LC/MS analysis, samples were cleaned up using a C18 spin column to remove impurities and enhance column longevity.

Analytical columns

Agilent ZORBAX RRHD Eclipse Plus C18 column, 2.1 × 150 mm, 1.8 µm (part number 959759-902)

Agilent Altura ZORBAX Eclipse Plus C18 inert column, 2.1 × 150 mm, 1.8 µm (part number 204215-308)

Instruments

LC/MS analysis was conducted using the following instrument configuration:

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler with sample thermostat (G7137A)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6549AA)

Table 1. LC parameters.

Agilent 1290 Infinity II Bio LC System	
Parameter	**Value**
Mobile Phase A	Water with 0.1% formic acid
Mobile Phase B	Acetonitrile with 0.1% formic acid
Column Temperature	60 °C
Flow Rate	0.4 mL/min
Sampler Temperature	8 °C
Gradient 1 for Synthetic Peptides	Time (min) %B 0.00 2 1.00 2 7.00 38 7.10 80 9.00 80 9.10 2 14.0 2
Gradient 2 for Herceptin Tryptic Digest	Time (min) %B 0 2 2 2 42 42 43 80 47 80 48 2 55 2

Table 2. MS parameters.

Agilent 6545 XT AdvanceBio LC/Q-TOF	
Parameter	**Value**
Source	Dual AJS Positive ESI
Source	
Drying Gas Temperature	250 °C
Drying Gas Flow	10 L/min
Sheath Gas Temperature	250 °C
Sheath Gas Flow	12 L/min
Nebulizer Pressure	25 psi
Capillary Voltage	3,500 V
Nozzle Voltage	0 V
Fragmentor	95 V
Skimmer	50 V

Parameter	Value
Acquisition – MS	
Acquisition Mode	Extended dynamic range (2 GHz)
Mass Range	*m/z* 100 to 3,000
Scan Rate	1 spectrum/sec
Reference Mass	121.0509, 922.0098
Acquisition – MS/MS	
Acquisition Mode	Auto MS/MS
Mass Range	*m/z* 100 to 3,000
Scan Rate	3 spectra/sec
Isolation Width (MS/MS)	Medium (~4 amu)
Max Precursors per Cycle	5
Threshold for MS/MS	6,000 counts and 0.001%
Precursor Charge	2+, 3+, > 3+
Target	50,000 counts/spectrum

Table 3. MS/MS collision energy parameters.

Ramped Collision Energy		
Charge	**Slope**	**Offset**
2	3.1	1
3	3.6	–4.8
> 3	3.6	–4.8

Table 4. Agilent MassHunter BioConfirm software, version 12.1 processing parameters.

Workflow and Sequencings		
Workflow	Protein digest	
Conditions	Reduced	
Sequences	Herceptin (directly alkylated), Trypsin	
Variable Modifications	Deamidation, G0, G0F, G0F-GlcNAc, G2, G2F, H5N3F1-1, methylation, oxidation (M, W), pyroGlu (E)	
Enzymes	Trypsin	
Find Peptides		
Biomolecule Filters	Display Molecules Containing MS/MS Scans	Enabled
Extraction	Use Peaks with Height	≥ 600 counts
Ion Species	Positive Ions	H^+, Na^+, K^+, NH_4^+
Charge State	Isotope Model	Peptides
	Limit Assigned Charge States to a Range of	1 to 5
Peak Filters (MS/MS)	Height Filters, Absolute Height Enabled	≥ 50 counts
Match Tolerances		
Mass Matching	± 5 ppm MS1, ± 20 ppm MS2	
Scoring	Warn if Score is < 7.00	
	Do Not Match if Score is < 5.00 (Sequence coverage)	
Matching Rules	MS/MS Fragmentation Type	CID
	Number of Missed Cleavages	2
	Max Number of Matches per Biomolecule	3
	Peptide Length Range	3 to 70
	Allow Only Either N-Term or C-Term Truncation	Selected
	Maximum Number of Mods	2

Results and discussion

Evaluating chromatographic improvements enabled by inert column hardware

To assess the performance advantages of the Altura ZORBAX Eclipse Plus C18 inert column hardware in peptide mapping workflows, we compared it with the SS ZORBAX RRHD Eclipse Plus C18 column. A synthetic peptide mixture containing mAb sequences was analyzed using the 1290 Infinity II Bio LC system coupled to a 6545XT AdvanceBio LC/Q-TOF.

Initial overlays of extracted ion chromatograms (Figure 1) showed comparable retention times across both columns. However, detailed inspection of individual peptides revealed notable differences in signal intensity, peak shape, and resolution (Figure 2). For example, the hydrophobic and basic peptide VVSVLTVLHQDWLNGK exhibited broader peaks and reduced signal intensity on the SS column compared to the Altura column. This suggests that metal-analyte interactions in SS hardware may affect sample recovery and/or chromatographic performance.

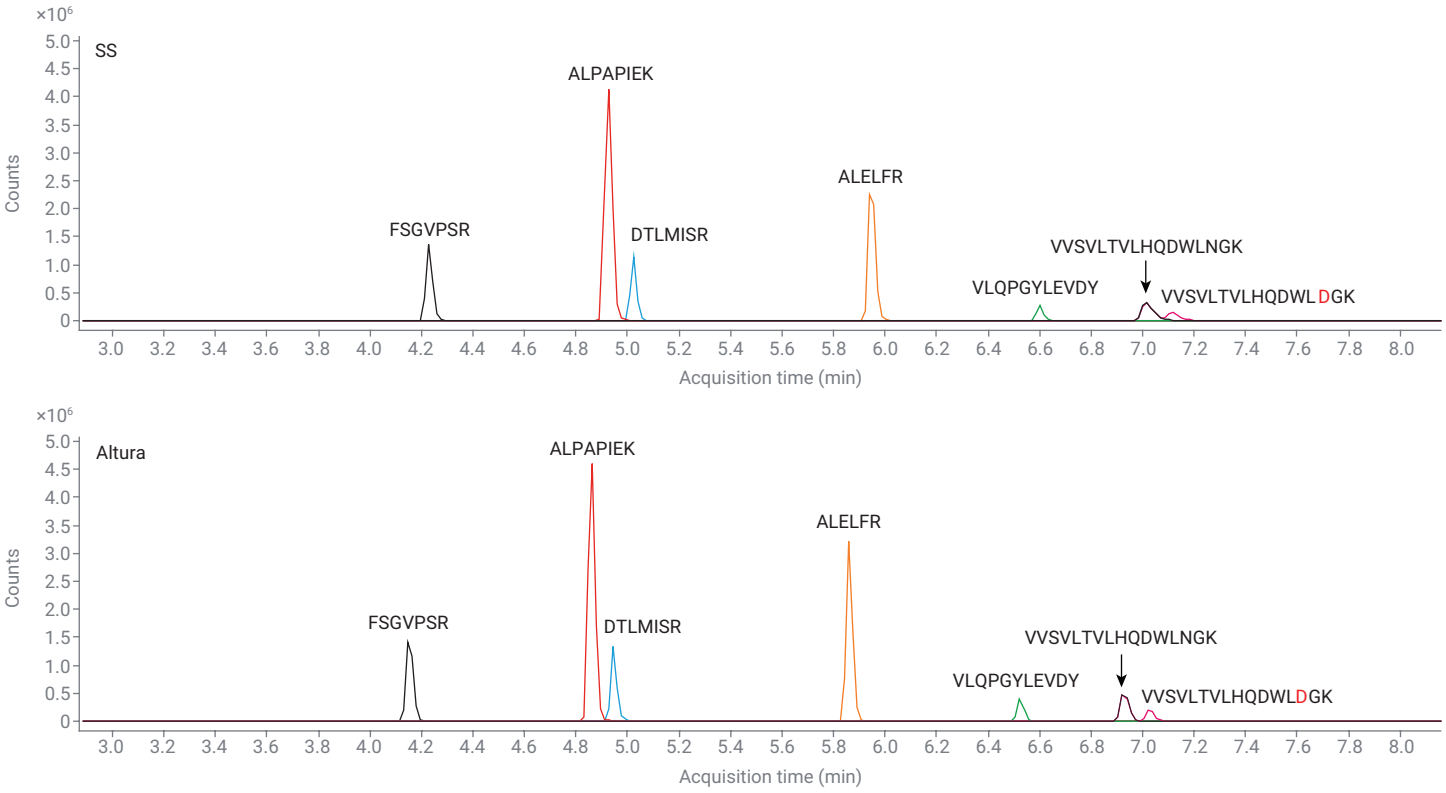


Figure 1. Chromatographic separation of synthetic monoclonal antibody peptides using stainless-steel (SS) and Agilent Altura ZORBAX Eclipse Plus C18 inert column hardware.

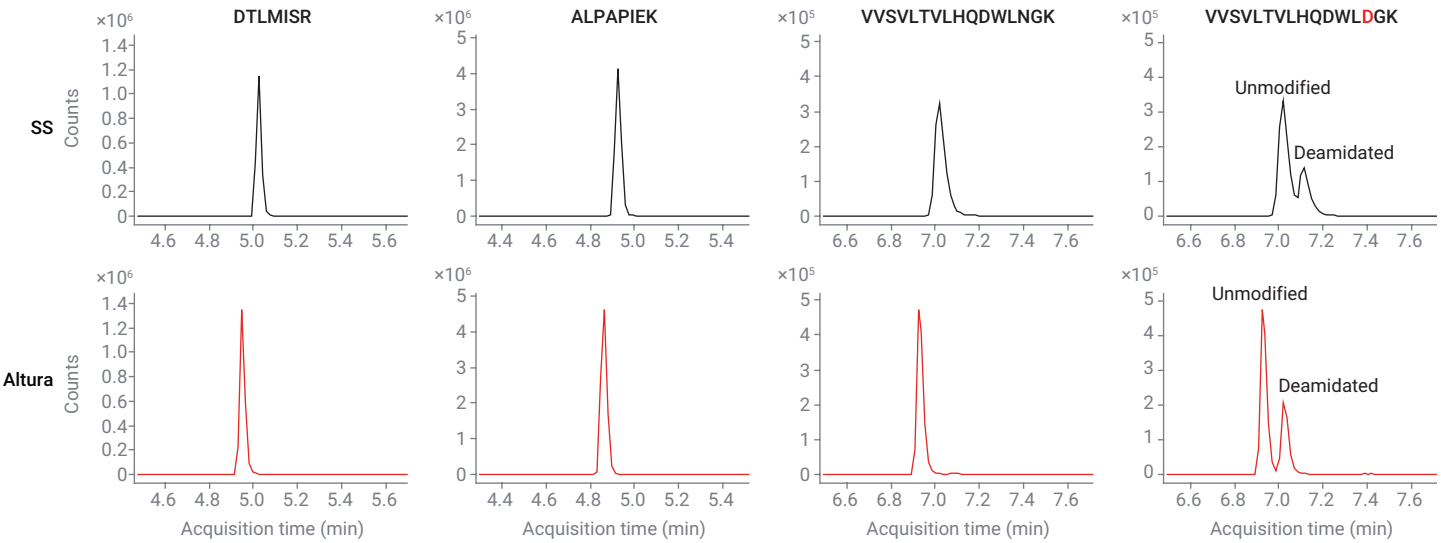


Figure 2. Improved peak shape and resolution of deamidated peptides using Agilent Altura ZORBAX Eclipse Plus C18 inert column hardware compared to stainless-steel (SS) column hardware.

Further analysis of the deamidated variant VVSVLTVLHQDWLDGK, where asparagine at position 14 is converted to aspartic acid, demonstrated improved separation from its unmodified counterpart when using the Altura column. This enhanced resolution is particularly valuable for detecting post-translational and chemical modifications such as deamidation, which represent critical quality attributes that must be characterized in biopharmaceutical development.

To determine the extent of the inert column hardware's benefit, new SS and Altura columns were subjected to one blank injection for quick conditioning, followed by 30 consecutive injections of the synthetic mAb peptide mixture (Figure 3). These results showed that the SS column required at least 10 injections to reach similar peak area recovery as the Altura column for ALPAPIEK and DTLMISR. For the more hydrophobic/basic peptide VVSVLTVLHQDWLNGK, it took nearly 20 injections for the SS column to match the Altura column's performance, highlighting the inert column's faster stabilization and superior reproducibility.

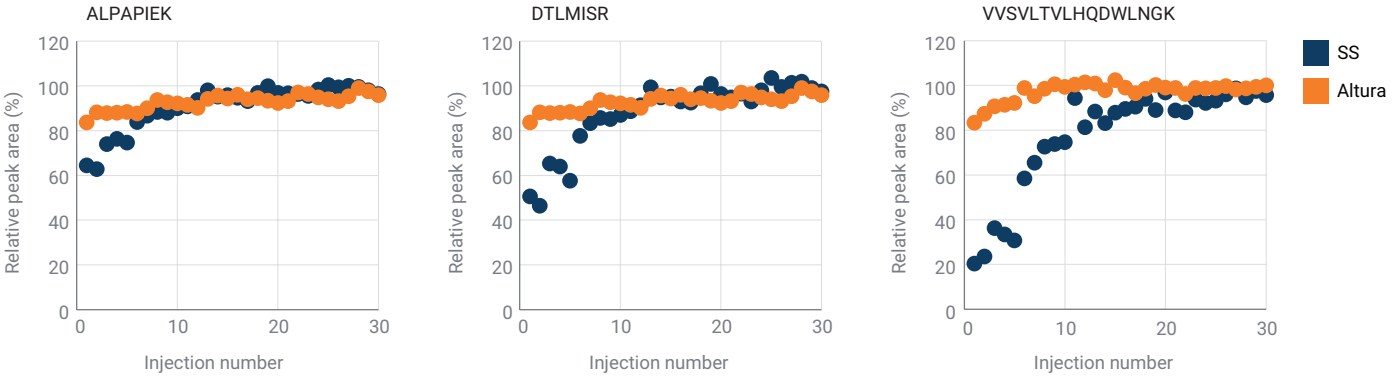


Figure 3. Quantitative comparisons for synthetic peptides using stainless-steel (SS) and Agilent Altura ZORBAX Eclipse Plus C18 inert columns. Relative peak area trends are plotted over 30 injections. Altura hardware shows faster stabilization and higher reproducibility compared to SS columns, particularly for hydrophobic and basic peptides.

Chromatographic performance indicators, such as tailing factors (TF), a metric used to quantify peak asymmetry, further supported these findings (Figure 4). TF for ALPAPIEK and DTLMISR were similar between the two columns, indicating comparable peak shapes. However, VVSVLTVLHQDWLNGK showed a significantly better TF with the Altura column (average TF = 1.3, n = 30) compared to the SS column (average TF = 1.7, n = 30). This correlates well with the qualitative observations in Figure 2 and suggests that inert hardware improves peak symmetry for challenging peptides.

Plotting TF across the 30 injections revealed that the SS column gradually improved with conditioning, eventually approaching the Altura column's performance. Nevertheless, poor initial peak shape on the SS column negatively impacted the separation of closely eluting analytes, such as the deamidated peptide VVSVLTVLHQDWLDGK from its unmodified counterpart. As shown in Figure 4C, the Altura column achieved greater resolution between these variants, enabling more confident identification in the chemical modification.

Despite the use of a relatively steep gradient (6% B/min over 6 minutes), the Altura column achieved superior separation. This finding indicates potential for further optimization by extending the gradient or reducing its slope to enhance resolution even more. Overall, these results demonstrate that the Altura ZORBAX Eclipse Plus C18 inert column not only improves chromatographic performance of PTM and chemical modification resolution but also supports higher sample throughput and better impurity profiling, making it a valuable tool for robust peptide mapping workflows.

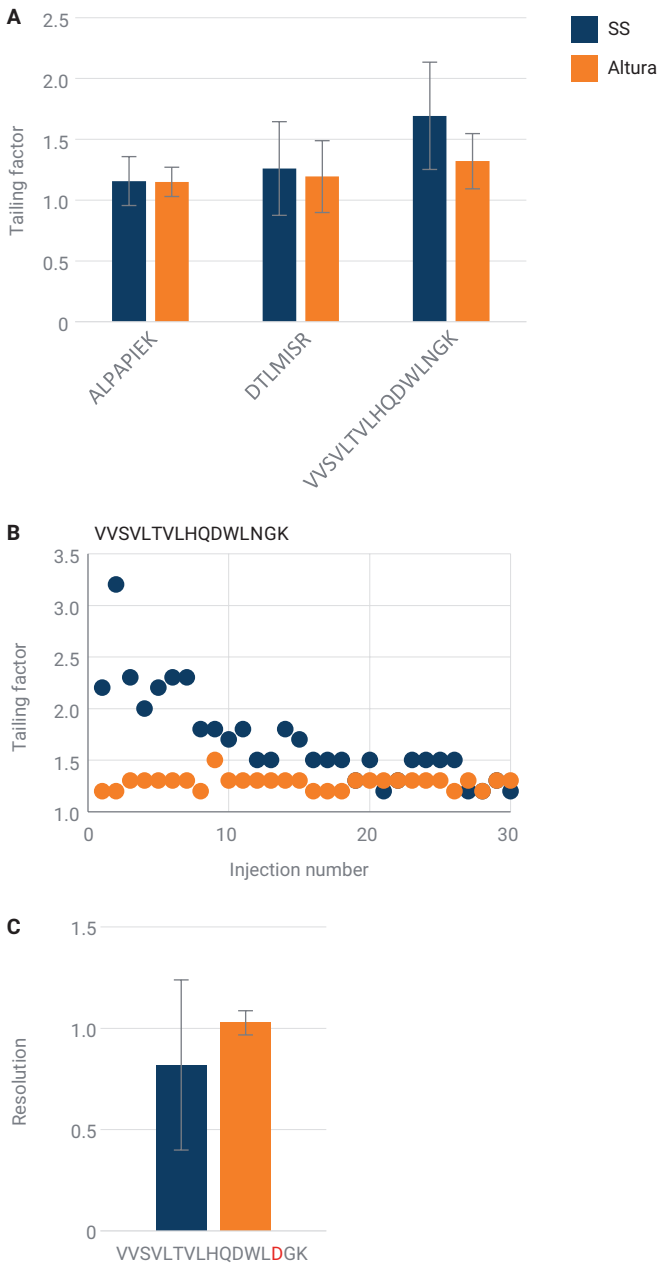


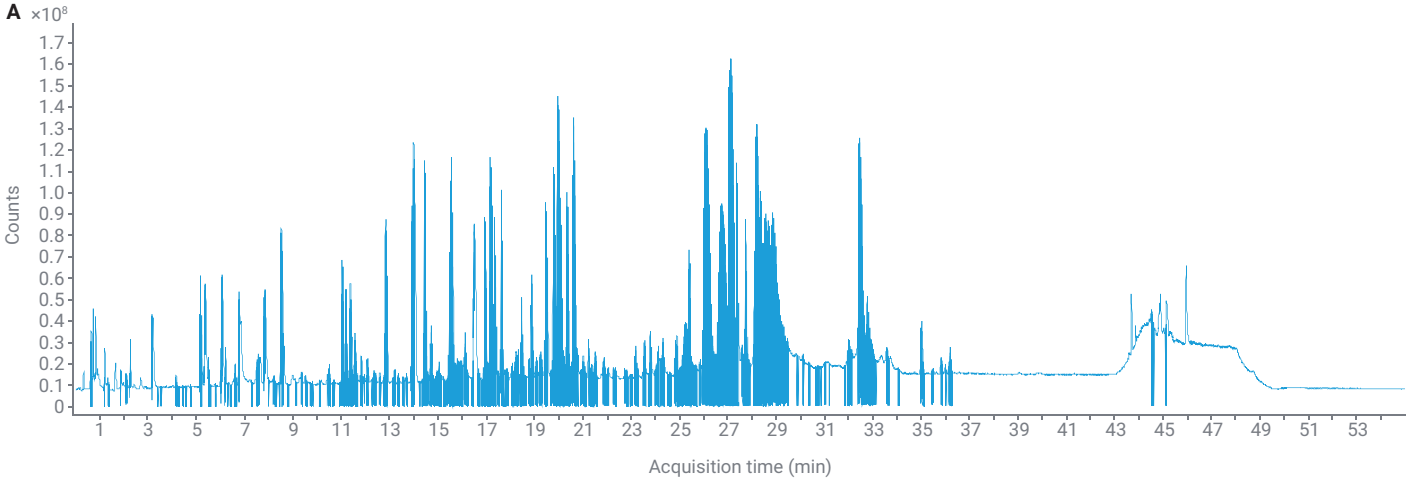
Figure 4. Chromatographic performance metrics for synthetic peptides using stainless-steel (SS) and Agilent Altura ZORBAX Eclipse Plus C18 inert columns. (A) Averaged tailing factors for ALPAPIEK, DTLMISR, and VVSVLTVLHQDWLNGK. (B) Tailing factor trends are plotted over 30 injections. (C) Resolution between the deamidated peptide pair VVSVLTVLHQDWLDGK and VVSVLTVLHQDWLNGK. The Altura column demonstrates improved peak symmetry and enhanced separation of closely eluting variants compared to SS hardware.

Peptide mapping of trypsin-digested Herceptin

Building on the demonstrated benefits of the Altura ZORBAX Eclipse Plus C18 inert column, we applied this technology to the peptide mapping of a trypsin-digested Herceptin sample. The digested sample was analyzed using LC/MS/MS with the Altura column, and the resulting total ion chromatogram (TIC) and sequence coverage are shown in Figure 5. The analysis yielded a sequence coverage of 87.03%, with the missing tryptic peptides being shorter and more hydrophilic in nature.

It is worth noting that the C18 cleanup step performed prior to LC/MS/MS analysis, as described in the Experimental section, may have contributed to the loss of these shorter hydrophilic peptides. This observation highlights an important consideration for future experimental designs aiming for comprehensive sequence coverage. A previous application note that did not use sample cleanup for the Herceptin digest was able to achieve a sequence coverage of ~98%.[4]

A



B Peptide mapping of Herceptin with Trypsin (sequence coverage: 87.03%)

Heavy chain

```
1    EVQLVESGGG LVQPGGSLRL SCAASGFNIK DTYIHWVRQA PGKGLEWVAR
51   IYPTNGYTRY ADSVKGRFTI SADTSKNTAY LQMNSLRAED TAVYYCSRWG
101  GDGFYAMDYW GQGTLVTVSS ASTKGPSVFP LAPSSKSTSG GTAALGCLVK
151  DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT
201  YICNVNHKPS NTKVDKKVEP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP
251  KDTLMISRTP EVTCVVVDVS HEDPEVKFNW YVDGVEVHNA KTKPREEQYN
301  STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ
351  VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV
401  LDSDGSFFLY SKLTVDKSRW QQGNVFSCSV MHEALHNHYT QKSLSLSPG
```

Light chain

```
1    DIQMTQSPSS LSASVGDRVT ITCRASQDVN TAVAWYQQKP GKAPKLLIYS
51   ASFLYSGVPS RFSGSRSGTD FTLTISSLQP EDFATYYCQQ HYTTPPTFGQ
101  GTKVEIKRTV AAPSVFIFPP SDEQLKSGTA SVVCLLNNFY PREAKVQWKV
151  DNALQSGNSQ ESVTEQDSKD STYSLSSTLT LSKADYEKHK VYACEVTHQG
201  LSSPVTKSFN RGEC
```

Figure 5. Peptide mapping of trypsin-digested Herceptin using the Agilent Altura ZORBAX Eclipse Plus C18 inert column. (A) Total ion chromatogram (TIC) for the LC/MS/MS run of trypsin-digested Herceptin. (B) Sequence coverage map showing 87.03% coverage with the identified peptides in dark blue text. The missing tryptic peptides (light blue text) have short and hydrophilic chemical features prone to sample loss during C18 cleanup step.

Figure 6 presents the CID MS/MS spectra for the peptide NTAYLQMDSLR in its deamidated form. Fragment ion data enabled confident assignment of the deamidation site at position 8, demonstrating the utility of MS/MS in characterizing post-translational and chemical modifications.

To emphasize the importance of chromatographic separation to characterize deamidated peptides, Figure 7 shows EICs for NTAYLQMNSLR in both its native and deamidated forms. Due to the small mass shift of 0.98 Da and the relatively low signal intensity of the deamidated variant, resolving these forms chromatographically is critical. Mass spectrometry alone cannot distinguish them effectively, as the deamidated peptide's monoisotopic peak (656.3221 *m/z*) overlaps with the second isotopic peak of the native peptide (monoisotopic peak: 655.8318 *m/z*, second isotopic peak: 656.3328 *m/z*) (16 ppm difference). Therefore, high-resolution chromatographic separation, enabled by inert column hardware, is essential for accurate identification and quantitation for peptide deamidation.

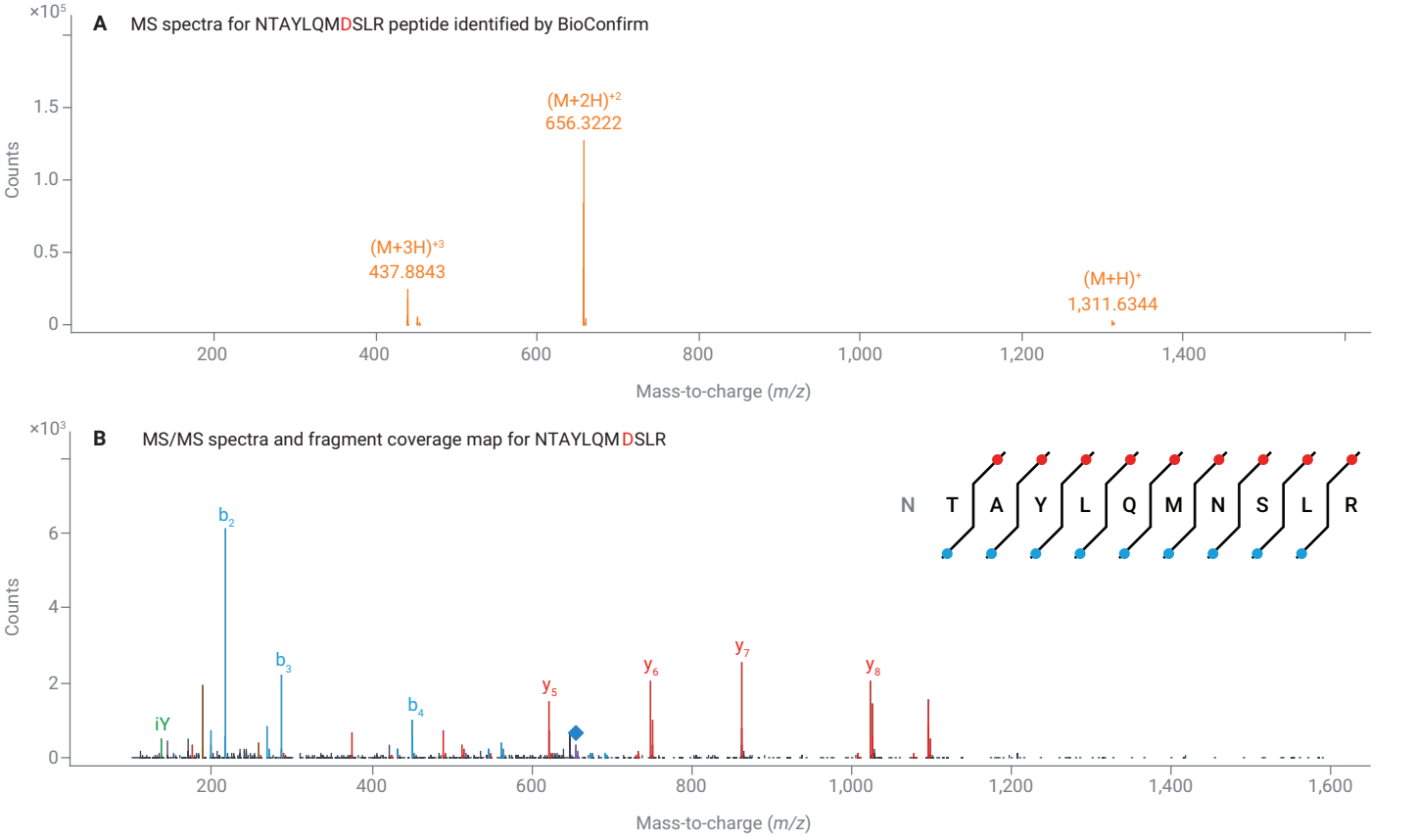


Figure 6. MS spectra and fragment coverage map for the NTAYLQMDSLR peptide. Fragmentation data were used to assign deamidation at position 8, using the Agilent MassHunter BioConfirm software.

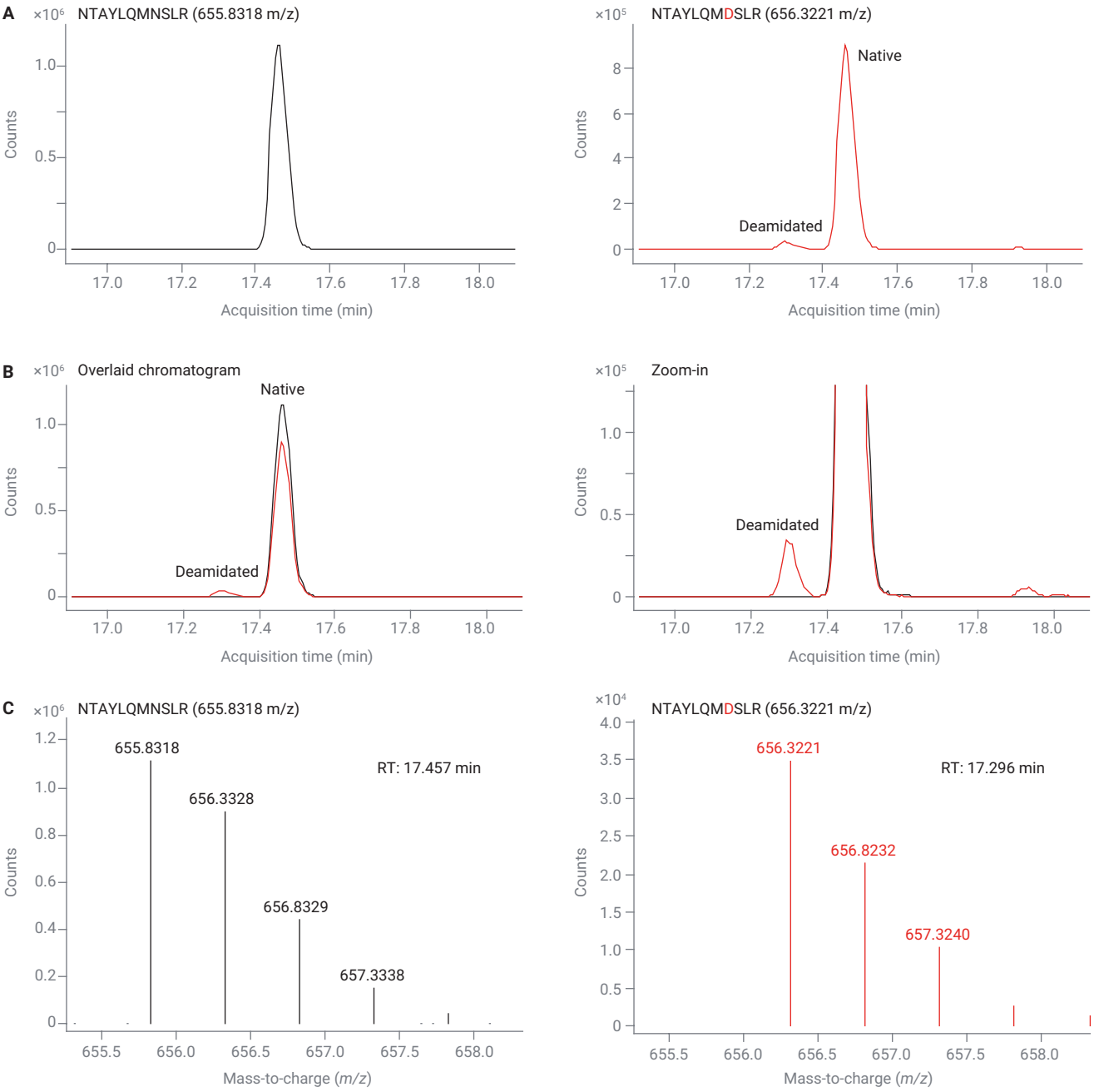


Figure 7. Chromatographic separation of NTAYLQMNSLR peptide in native and deamidated forms. (A) Extracted ion chromatograms (EICs) using precursor masses of the native (655.8318 *m/z*) and deamidated (656.3221 *m/z*) forms at 2+ charge state. (B) The individual chromatograms were overlaid to illustrate the chromatographic separation between the native and deamidated peptides, with the zoom-in chromatogram highlighting the separation of the low-level impurity. (C) Mass spectrum of the native and deamidated peptides are shown to illustrate the need to chromatographically resolve the deamidated form from the native one, otherwise, the mass spectra alone would not be able to distinguish the two forms if they were coeluting.

Conclusion

This study demonstrates the significant advantages of the Agilent Altura ZORBAX Eclipse Plus C18 inert column hardware for peptide mapping workflows, particularly in the analysis of mAb peptides and post-translational and chemical modifications. Compared to conventional SS column hardware, the Altura column consistently delivered improved peak shape, reduced variability, and enhanced resolution, especially for challenging hydrophobic and modified peptides.

Quantitative data from 30 consecutive injections revealed faster column stabilization and superior reproducibility with Altura hardware, while chromatographic metrics such as tailing factor and resolution confirmed its ability to better separate closely eluting species. These benefits were further validated in the peptide mapping of trypsin-digested Herceptin, where the Altura column enabled confident identification of deamidated variants.

Overall, the Altura ZORBAX Eclipse Plus C18 inert column together with the 1290 Infinity II Bio LC and 6545XT AdvanceBio LC/Q-TOF systems offer a robust and reliable solution for high-throughput peptide mapping and characterization of modifications, supporting both analytical precision and operational efficiency in biopharmaceutical development.

References

1. Hsiao, J.; *et al.* Pushing the Boundaries of Chromatographic Separation with Inert HPLC Column Hardware. *Agilent Technologies application note*, 5994-8618EN, **2025**.
2. Vanluchene, H.; *et al.* mRNA Mapping with IO-RPLC-MS on a Low-Adsorption Flow Path. *Agilent Technologies application note*, 5994-8055EN, **2025**.
3. Feith, A. Robust and Reliable Peptide Mapping. *Agilent Technologies application note*, 5994-2718EN, **2024**.
4. Sciuto, S. Peptide Mapping of Trastuzumab Tryptic Digests on an Agilent 6545XT AdvanceBio LC/Q-TOF. *Agilent Technologies application note*, 5994-7969EN, **2024**.

www.agilent.com

DE-010885

Printed in the USA, November 18, 2025
5994-8776EN

Agilent
Trusted Answers