Application Note

Biopharma

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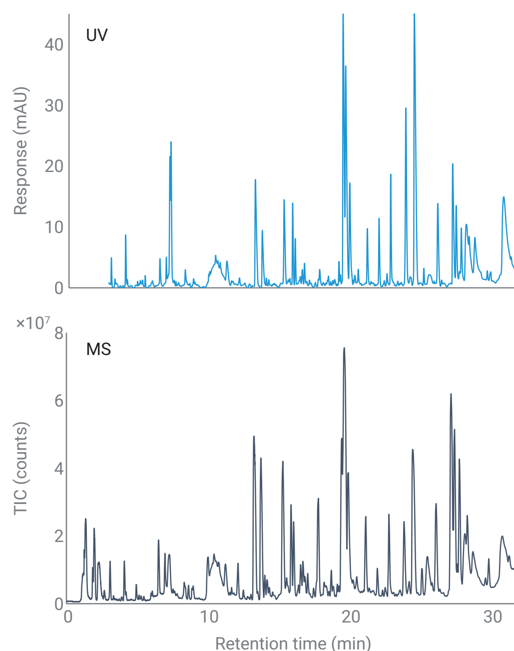
Robust and Reliable Peptide Mapping

The Agilent 1290 Infinity II Bio LC System as the new platform for UV and MS-based primary structure and PTMs analysis of mAbs



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Abstract

Peptide mapping is the gold standard for elucidating the primary structure of monoclonal antibodies (mAbs). However, the key to successful peptide mapping is a robust and reliable LC system for high-quality peptide separation. In this application note, we present the Agilent 1290 Infinity II Bio LC as the system of choice for peptide mapping. Recreation of a published comprehensive peptide-mapping method for the NISTmAb showed exceptionally good relative retention time deviations below 0.1% even for very shallow gradients. Further method development decreased the total run time by 60%, keeping the excellent relative standard deviations and peak capacity values. Additionally, the 1290 Infinity II Bio LC was connected directly to the Agilent 6545XT AdvanceBio LC/Q-TOF as an example of a method development setup, facilitating easy method transfer throughout the biopharmaceutical production chain.

Introduction

Peptide mapping is a widely used technique for analyzing the primary structure and post-translational modifications (PTMs) of biopharmaceuticals in today's industrial biotechnology. Typically, bottom-up approaches are employed by denaturation, alkylation, and digestion of a mAb. Subsequently, resulting peptides are separated by HPLC or UHPLC using reversed-phase or even hydrophilic interaction liquid chromatography, in some cases. Detection is either carried out with mass spectrometry (MS), to identify a drug substance, or ultraviolet (UV) absorbance in quality control (QC) environments, by comparison of the chromatographic profile to a reference map. Peptide mapping can be used as part of the acceptance criteria for the evaluation of biological products, which is described in ICH Guideline Q6B.[1] By using LC/MS or UV, changes in the peptide map—for example, increased oxidation or deamidation,[2] the appearance of new sequence variants,[3] or changes in the glycan composition[4]—can be evaluated. Therefore, precision and robustness, especially when using a UV detector, are of utmost importance to release and develop safe and potent biopharmaceuticals.

This application note showcases the new 1290 Infinity II Bio LC as a novel platform for peptide mapping. Exploiting the high-precision, binary Agilent 1290 Infinity II Bio High-Speed Pump and a biocompatible, iron-free flow path, the system is especially suited to biomolecules like peptides, proteins, and metabolites.

Experimental

Equipment

The Agilent 1290 Infinity II Bio LC System coupled to the Agilent 6545XT AdvanceBio LC/Q-TOF comprised the following modules:

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116A) equipped with a Standard Flow Quick Connect Bio Heat Exchanger (G7116-60071) and two Agilent Thermal Equilibration Devices (G7116-60013)
- Agilent 1290 Infinity II Variable Wavelength Detector (VWD) (G7114B), equipped with a Bio Micro Flow Cell VWD, 3 mm, 2 µL, RFID.
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6545XT)

Software

- Agilent MassHunter workstation data acquisition (B.09.00)
- Agilent MassHunter Qualitative Analysis (B.10.00)
- Agilent MassHunter BioConfirm (B.10.00)

Columns

- Agilent AdvanceBio Peptide Mapping, 2.1 × 250 mm, 2.7 µm (part number 651750-902)
- Agilent AdvanceBio Peptide Mapping Fast Guards, 2.1 × 5 mm, 2.7 µm (part number 851725-911)
- Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm (part number 959759-902)
- Agilent ZORBAX RRHD Eclipse Plus C18 Fast Guards, 2.1 × 5 mm, 1.8 µm (part number 821725-901)

Chemicals

LC-grade acetonitrile, ammonium bicarbonate, *tris*(2-carboxyethyl) phosphine, and 2-iodoacetamide were purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Formic acid was purchased from VWR (Darmstadt, Germany). Trypsin (porcine, mass spectrometry-grade) was obtained from G-Biosciences (St. Louis, USA).

Sample preparation

0.8 mg of the Agilent-NISTmAb (part number 5191-5744) in 100 µL ammonium bicarbonate (100 mM) was denatured and reduced by the addition of 2 µL of *tris*(2-carboxyethyl)phosphine (TCEP, 200 mM) and incubated at 60 °C for 1 hour. After the alkylation with 4 µL of 2-iodoacetamide (IAM, 200 mM, 1 hour at RT), quenching of excess IAM with 2 µL of TCEP (1 hour at RT), and subsequent dilution with 0.8 mL of 25 mM ammonium bicarbonate, the enzyme trypsin was added (20:1, NISTmAb to trypsin w/w). After the overnight digestion at 37 °C, the pH of the resulting suspension was decreased below pH 4 by the addition of 2 µL of formic acid.

Table 1. Method A: Comprehensive NISTmAb peptide-mapping method adapted from Mouchahoir & Schiel, 2018.[5]

Parameter	Value
Column	Agilent AdvanceBio Peptide Mapping, 2.1 × 250 mm, 2.7 µm + Fast Guard 2.1 × 5 mm
Solvent	A) Water + 0.1% formic acid B) Acetonitrile + 0.1% formic acid
Gradient	0.00 min – 2% B 110.00 min – 45% B 110.01 min – 97% B 125.00 min – 97% B 125.01 min – 2% B 150.00 min – 2% B
Flow rate	0.200 mL/min
Temperature	40 °C with thermal equilibration devices installed
Detection	VWD: 214 nm, 10 Hz/MS: see Table 3
Injection	Injection Volume: 15 µL Sample temperature: 4 °C Wash: 3 s in water (Flush Port)

Table 2. Method B: Optimized and shortened peptide-mapping method.

Parameter	Value
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 2.1 × 150 mm, 1.8 µm + Fast Guard 2.1 × 5 mm
Solvent	A) Water + 0.1% formic acid B) Acetonitrile + 0.1% formic acid
Gradient	0.00 min – 2% B 44.00 min – 45% B 44.01 min – 97% B 50.00 min – 97% B 50.01 min – 2% B 60.00 min – 2% B
Flow rate	0.300 mL/min
Temperature	40 °C with thermal equilibration devices installed
UV detection	VWD: 214 nm, 10 Hz/MS: see Table 3
Injection	Injection volume: 15 µL Sample temperature: 4 °C Wash: 3 s in water (Flush Port)

Table 3. Source and MS parameters for the iterative MS/MS analysis of peptides.

Parameter	Value
Instrument	Agilent 6545XT AdvanceBio LC/Q-TOF
Gas Temperature	325 °C
Drying Gas Flow	13 L/min
Nebulizer	35 psig
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Acquisition Mode	Extended dynamic range (2 GHz)
Mass Range	*m/z* 100 to 1,700
Acquisition Rate	8 spectra/sec
Auto MS/MS Range	*m/z* 50 to 1,700
Isolation Width	Narrow (~*m/z* 1.3)
Precursors/Cycle	10
Collision Energy	Charge 2: 3.1 (slope) and 1 (offset) Charge 3 and >3: 3.6 (slope) and –4.8 (offset)
Precursor Threshold	1,000 counts and 0.001%
Active Exclusion	After one spectrum for 0.2 min
Scan Speed Based On Precursor Abundance	Yes, 25,000 counts/spectrum
MS/MS Accumulation Time Limit	Yes
Purity	Stringency: 100%/cut-off: 30%
Isotope Model	Peptides
Sort Precursors	Charge state then abundance

Results and discussion

Tryptic digests of protein biopharmaceuticals such as mAbs present a highly complex mixture of numerous peptides. To determine and analyze the primary structure of these biopharmaceuticals, very long and shallow gradients are deployed, which can range up to several hours' run time, putting high demands on the instrumentation. Showcasing the suitability of the 1290 Infinity II Bio LC for this challenging analysis, we chose to recreate an LC/UV and MS method previously published by the National Institute of Standards and Technology (NIST) for the tryptic digest of the NISTmAb.[5] For this, the AdvanceBio Peptide Mapping column with a length of 250 mm was used with a total method run time of 2.5 hours (Method A, Table 1). Additionally, a second LC method was developed to decrease run time by exploiting the sub-2 µm particles of the ZORBAX RRHD Eclipse Plus column (Method B, Table 2). Figure 1 shows the chromatograms of both methods detected with the Agilent 1290 Infinity II Variable Wavelength Detector (VWD).

Similar peptide patterns can be observed in both chromatograms. However, most analytes could be eluted after 30 minutes with Method B compared to 80 minutes with the originally published NIST Method A. As a consequence, the total run time could be decreased by 60%. To systematically evaluate the precision and robustness of the 1290 Infinity II Bio LC, eight peaks were chosen in both methods. Subsequently, retention time standard deviations were calculated based on 10 consecutive injections (Figure 2).

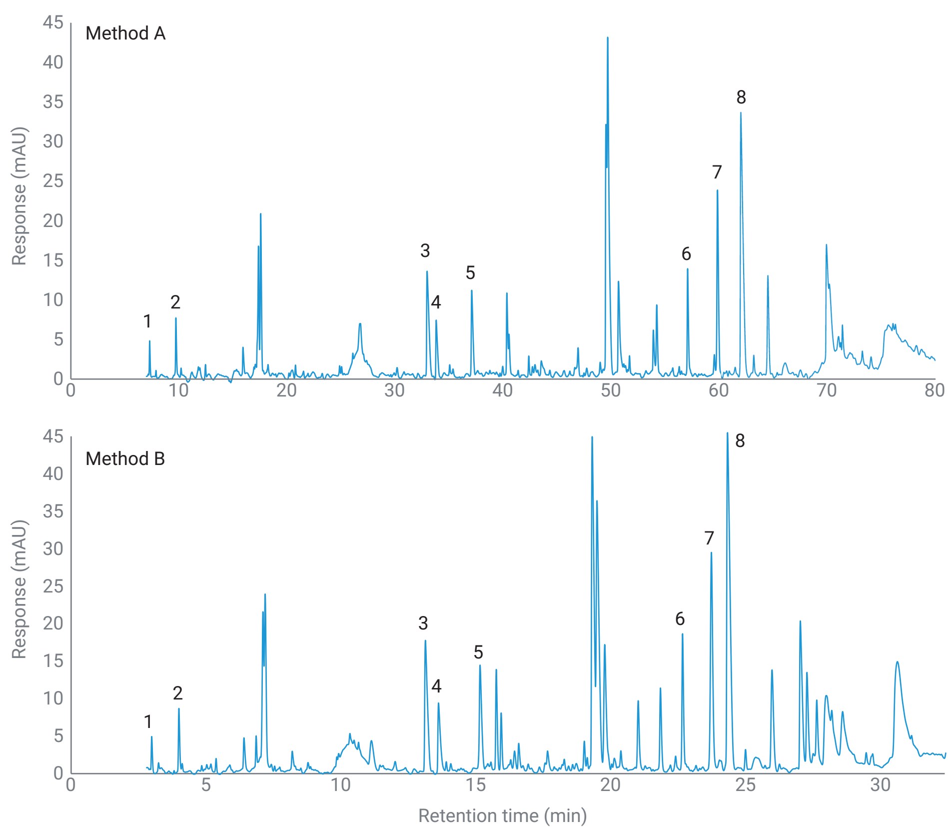
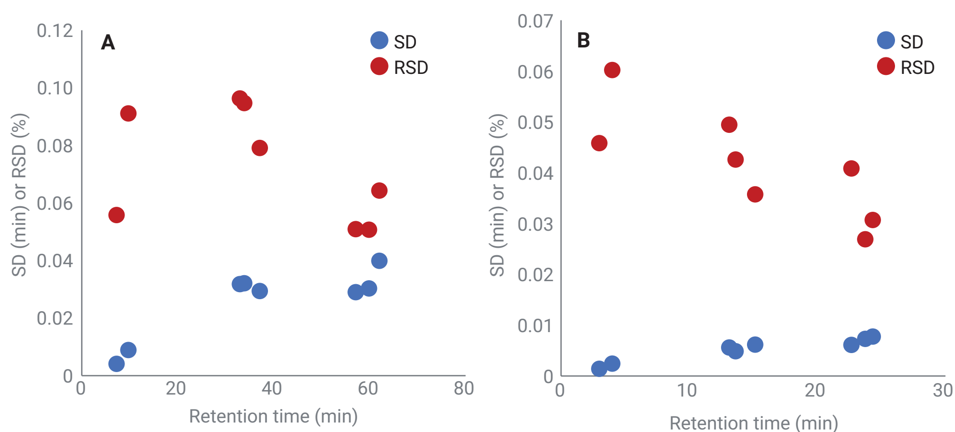


Figure 1. Chromatograms of a tryptic digest of the NISTmAb separated by the Agilent 1290 Infinity II Bio LC with Methods A and B. Eight peaks were picked for subsequent retention time precision and peak capacity calculations.



	Gradient Slope (%B/min)	Ø SD (min)	Ø RSD (%)	$P_{C,4\sigma}$ (–)
Method A	0.39	0.026	0.073	428
Method B	0.98	0.005	0.039	348

Figure 2. Absolute (SD) and relative (RSD) retention time precision values of Methods A and B acquired with the Agilent 1290 Infinity II Bio LC. The gradient slope and peak capacity are depicted in the table.

Methods A and B both show relative retention time deviations below 0.1%, displaying the exceptional performance of the 1290 Infinity II Bio High-Speed Pump even at very shallow gradient slopes of 0.39 and 0.98% B/min, respectively. To evaluate the comprehensive separation character of the LC methods, 4σ peak capacities were calculated as a measure of the quality of the separation. Due to the extended run time of Method A, the corresponding peak capacity value was the highest with 428. However, combining the outstanding average RSD of 0.039% with a high peak capacity of 348, Method B stands as a serious alternative, with a greatly decreased run time compared to the published peptide-mapping method provided by the NIST.

A typical workflow for peptide mapping in a biotechnological environment uses a UV and MS detector in sequence. With this setup, method development can be done with both detectors, using the MS for the identification of peptides. After establishing the method, the analysis can be easily transferred to the UV detector for high-throughput analysis in a QC environment. To demonstrate this case, the 1290 Infinity II Bio LC was directly connected to the 6545XT AdvanceBio LC/Q-TOF, and the tryptic digest of the NISTmAb was reanalyzed with Method B. MS detection was carried out in iterative MS/MS mode as shown in Table 3. Resulting chromatograms are depicted in Figure 3.

Even though no special measures were taken against peak broadening the resolution remained more than sufficient for reliable MS detection. Identification and confirmation of the primary structure of mAbs can conveniently be carried out by using the AgilentMassHunter BioConfirm software. Comparing the identified peptides on the MS and/or MS/MS level with a reference sequence of the biopharmaceutical of choice, PTMs can be analyzed and quantified relatively. With this approach, the so-called PENNY peptide (GFYPSDIAVEWESNGQPENNYK)[6] and the corresponding deamidated isoform could be identified. The PENNY peptide is part of the conserved region (Fc) shared by nearly all human or humanized mAbs, which can be used as decent indicator for induced deamidation. A zoomed-in view of these peptides is depicted in Figure 4.

After identifying the peptides, relative quantification can also be carried out by UV detection in this case, owing to the great separation capability of the optimized peptide-mapping Method B in combination with the excellent retention time precision of the 1290 Infinity II Bio LC.

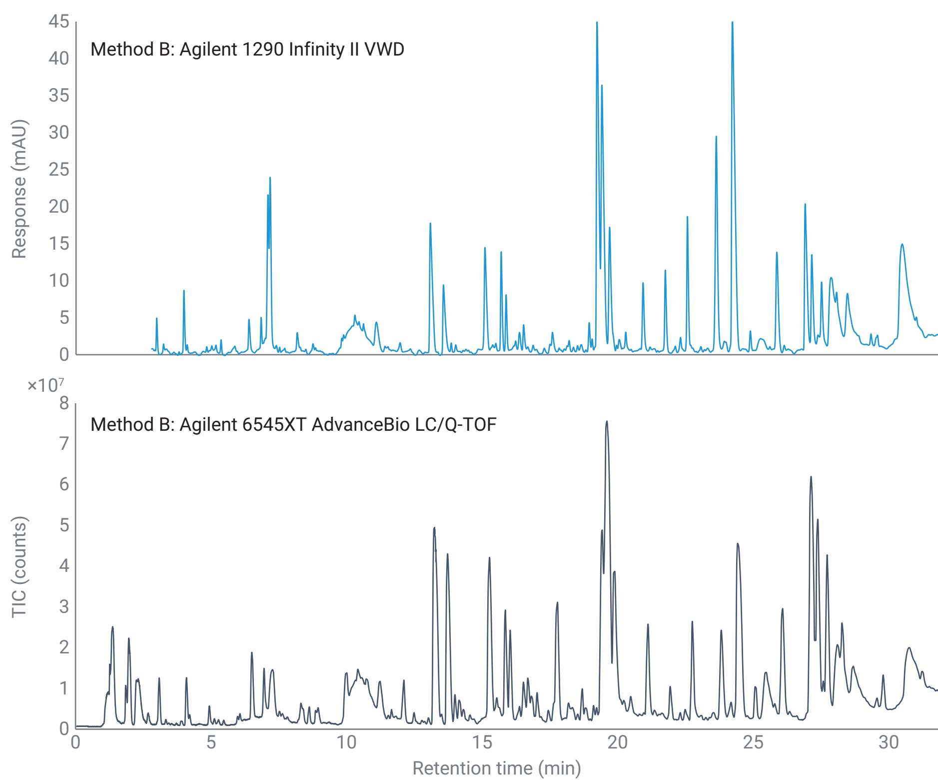


Figure 3. Chromatograms of a tryptic digest of the NISTmAb detected in sequence with the Agilent 1290 Infinity II VWD equipped with the biocompatible micro flow cell (upper) and the Agilent 6545XT AdvanceBio LC/Q-TOF (lower).

Conclusion

Critical quality attributes (CQA) such as sequence or glycosylation variants, oxidation, and deamidation can be analyzed by peptide mapping. However, it is mandatory that the used method and instrumentation are robust and reliable to deliver the best results possible. In this application note, we showed that the new 1290 Infinity II Bio LC can live up to these high expectations. Retention time precision deviations below 0.1% could be routinely achieved by recreating a comprehensive published peptide-mapping method for the NISTmAb. By optimizing this method, the total run time could be decreased by 60% without compromising the excellent precision and separation quality thanks to the 1290 Infinity II Bio High-Speed Pump. To present the usability in a method development environment, it was shown that the capability to connect the 1290 Infinity II Bio LC directly to a 6545XT AdvanceBio LC/Q-TOF enables the straightforward method transfer to a high-throughput QC environment. To sum up, the 1290 Infinity II Bio LC can be the new platform for UV and MS-based primary structure and PTMs analysis of mAbs.

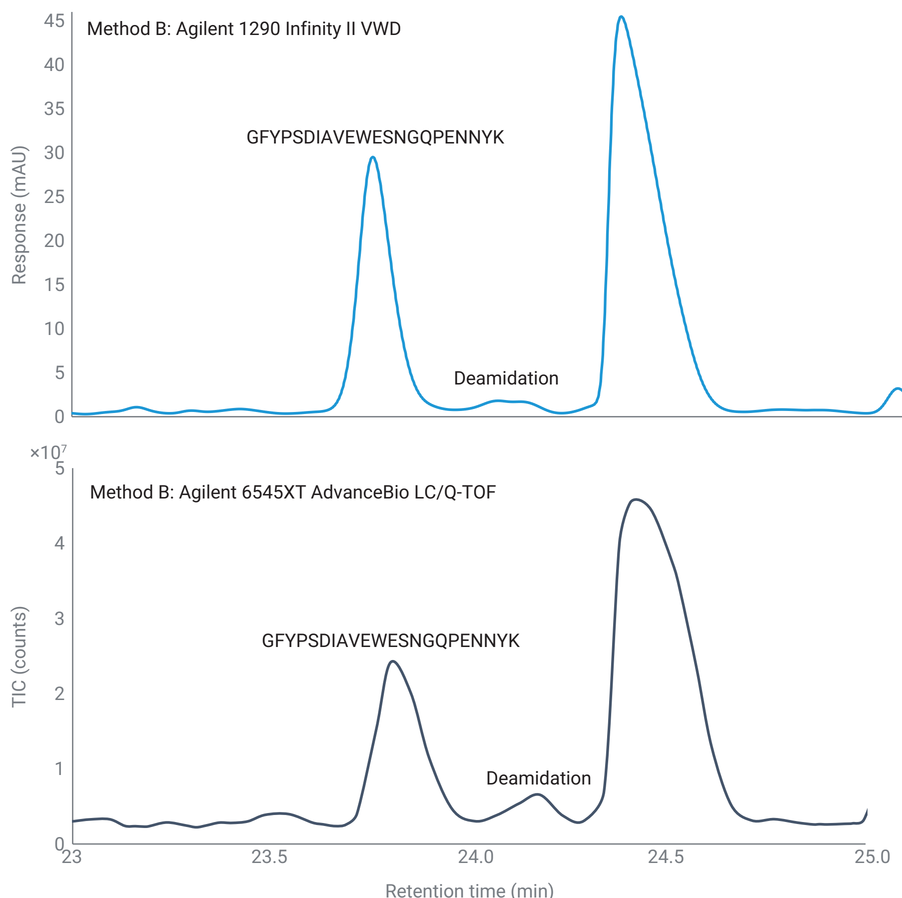


Figure 4. Magnified view of the previous chromatogram to highlight the separation of the PENNY peptide (GFYPSDIAVEWESNGQPENNYK) and corresponding deamidated isoform.

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