Application Note

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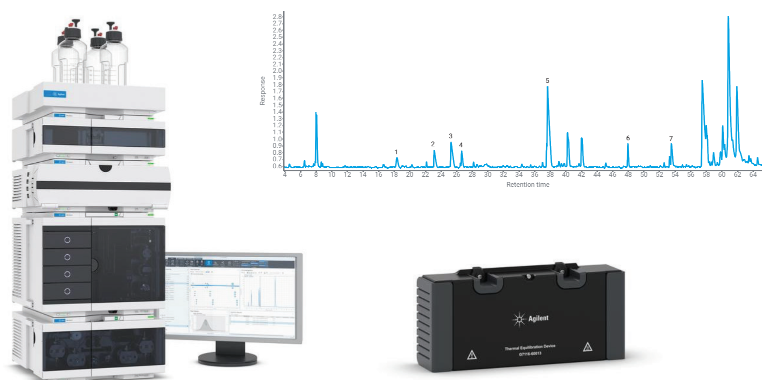
Peptide Mapping at Elevated Temperatures

Method Development with the Agilent 1290 Infinity II Bio LC System and Agilent Thermal Equilibration Device



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Abstract

Peptide mapping is an essential method to analyze the primary structure and determine the post-translational modifications (PTMs) of monoclonal antibodies (mAbs) and therapeutic proteins. It is also a crucial method in quality control (to ensure batch quality) and in research and development (for characterization). For these reasons, peptide mapping is indispensable to the biopharmaceutical industry. The complexity of the samples makes high resolution and a robust peptide-mapping method even more important. This application note demonstrates the resolution and robustness made possible with an Agilent Thermal Equilibration Device (TED) in combination with an Agilent 1290 Infinity II Bio LC at elevated temperatures.

Introduction

MAbs are an important class of therapeutics because of their specificity, long serum half-life, favorable toxicology profile, and their established manufacturing process.[1] Therapeutic mAbs are heterogeneous, and they are prone to a wide range of PTMs like oxidation, deamidation, isomerization, glycosylation, and glycation.[1] These modifications can occur during expression, purification, or storage, and they are classified as quality attributes. According to regulatory guidelines, some of these characteristics are declared critical quality attributes (CQAs).[1] The CQAs were assessed in terms of their risk to safety and efficacy. To ensure product quality and process consistency, these CQAs must be monitored during clinical development and after commercialization.[1]

To determine the CQAs of mAbs, peptide mapping is one of the most essential methods to characterize a protein, especially in research. The technique is also gaining importance in quality assurance (QA) and quality control (QC). Nowadays, peptide mapping is becoming more important for industrial biotechnology, not only for research but also for QC in particular. A bottom-up approach is used most often, and typically begins with denaturation followed by alkylation and digestion of the protein. Subsequently, the resulting peptides are separated by HPLC or UHPLC using reversed-phase liquid chromatography (RPLC). Detection can either be performed by optical detection to compare with a reference material in a QC approach, or with mass spectrometry (MS) to characterize and identify a protein.[2]

RPLC is a powerful method for peptide-mapping analysis.[3] Several studies have shown the impact of separation conditions such as ion-pairing reagent type and concentration, gradient slope, and column temperature on peptide peak shape, retention time, and separation selectivity.[3] The impact of an ion-pairing reagent on retention time and selectivity is well known, but the impact of other separation parameters such as temperature is less obvious.[3] Column temperature plays two important roles in RPLC: control of retention and control of selectivity.[4] Method optimization is usually erratic.[3]

This application note demonstrates the high resolution and robustness achievable with a TED together with the 1290 Infinity II Bio LC System, especially in peptide mapping at higher temperatures.

Experimental

Equipment

The Agilent 1290 Infinity II Bio LC System 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 (G7116B) 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

Software

Agilent OpenLab CDS Version 2.6 or later versions

Columns

- Agilent AdvanceBio Peptide Mapping, 2.1 × 150 mm, 2.7 µm (part number 653750-902)
- Agilent Advance Bio Peptide Mapping Fast Guard, 2.1 × 5 mm, 2.7 µm (part number 851725-911)

Chemicals

LC-grade acetonitrile was purchased from Merck (Darmstadt, Germany). Ammonium bicarbonate, tris(2-carboxyethyl) phosphine, and 2-iodoacetamide were purchased from Sigma-Aldrich (Steinheim, 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 (bovine, mass spectrometry-grade) was purchased from G-Biosciences (St. Louis, MO, USA).

Sample preparation

Agilent-NISTmAb (0.8 mg, part number 5191-5744) was denatured and reduced by mixing it with 100 µL ammonium bicarbonate (100 mM), 2 µL of tris(2carboxyethyl) phosphine (TCEP, 200 mM), and incubating for 1 hour at 60 °C. Subsequently, alkylation was performed by adding 4 µL of 2-iodoacetamide (IAM, 200 mM) and incubating for 1 hour at room temperature (RT) in darkness. To quench the excess of IAM, 2 µL TCEP was added and incubated 1 hour at RT. Afterwards, the sample was diluted with 0.8 mL ammonium bicarbonate (25 mM) and the enzyme trypsin was added (20:1, NISTmAb to trypsin w/w). After overnight digestion at 37 °C, the pH of the resulting suspension was lowered to below pH 4 by addition of 2 µL formic acid to quench trypsin activity.

Methods

Table 1. Chromatographic conditions.

Parameter	Value
Column	Agilent AdvanceBio Peptide Mapping, 2.1 × 150 mm, 2.7 µm with Agilent AdvanceBio Peptide Mapping Fast Guard, 2.1 × 5 mm
Solvent	A: Water + 0.1% formic acid B: Acetonitrile + 0.1% formic acid
Gradient	Time (min) %A %B 0.00 98 2 110.00 55 45 110.01 03 97 125.00 03 97 125.01 98 02 150.00 98 02 Stop time: 150 min Post time: 0 min
Flow Rate	0.200 mL/min
Temperature	80 °C
Detection	VWD: 214 nm, 10 Hz
Injection	Injection Volume: 15 µL Sample temperature: 8 °C Wash: 3 s in water (flush port)

Results and discussion

Two different experimental setups were used for the analysis of the Agilent-NISTmAb digest, with varying configurations of the 1290 Infinity II Multicolumn Thermostat (MCT). Both setups include a standard-flow biocompatible heat exchanger in combination with an AdvanceBio Peptide Mapping, 2.1 × 150 mm, 2.7 µm column with an AdvanceBio Peptide Mapping Fast Guard, 2.1 × 5 mm precolumn. There was only one difference between these experiments. In the first experiment, the 1290 Infinity II MCT was fully equipped with two TEDs, while in the second experiment, a TED was not installed.

Seven consecutive injections of the Agilent-NISTmAb sample were measured with each setup at 80 °C and by analyzing seven peaks. These peaks were chosen because of their wide distribution in the chromatogram.

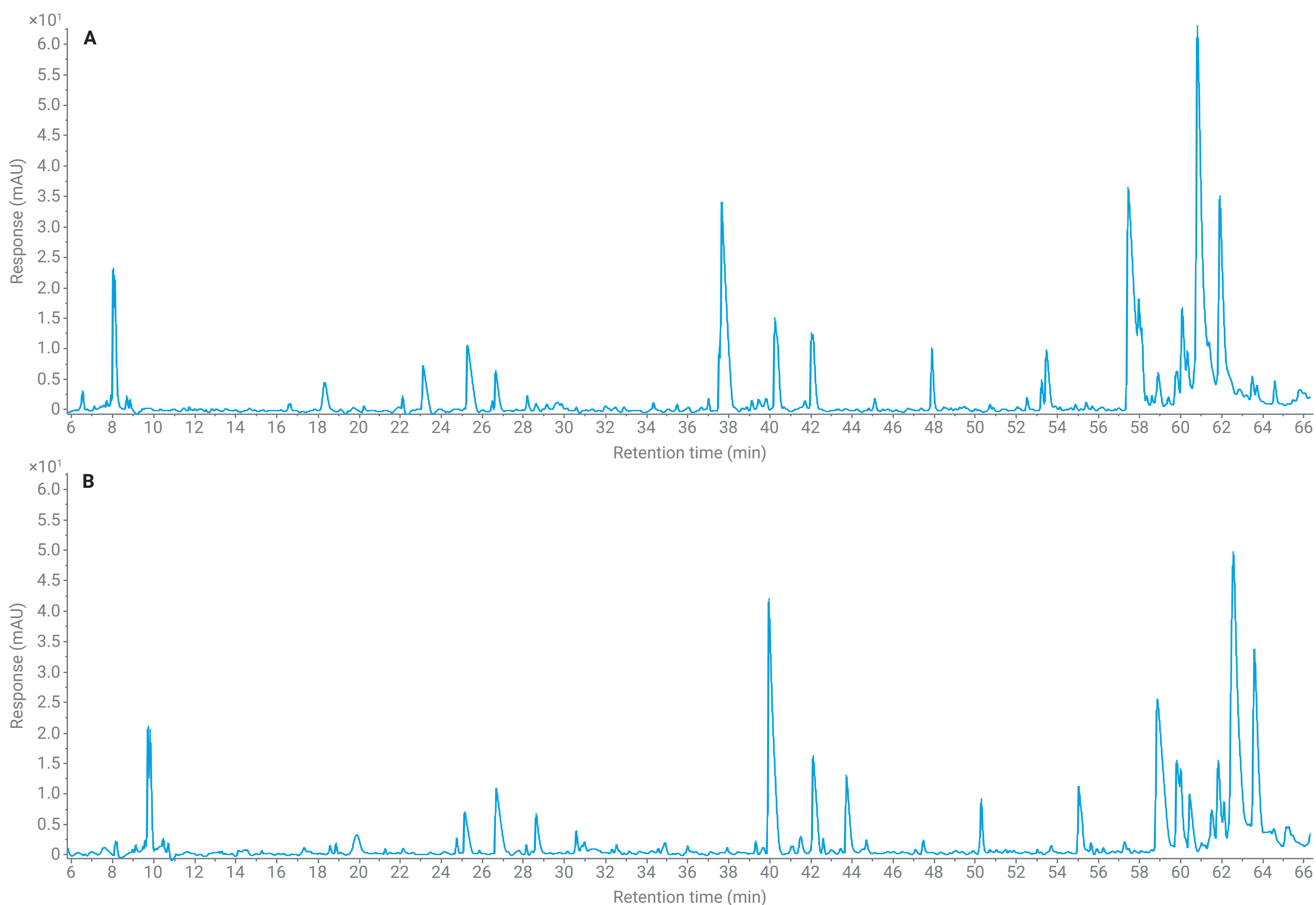

Figure 1. Chromatogram of a tryptic digest of the Agilent-NISTmAb separated by the Agilent 1290 Infinity II Bio LC at 80 °C with (A) and without (B) the Agilent Thermal Equilibration Device. Seven peaks were picked to determine precision of retention time.

Table 2 shows the relative standard deviation (RSD) of retention time to highlight the performance and compare the reproducibility of the two experiment setups at 80 °C.

Table 2. Relative standard deviation (RSD) values of retention time (RT) for the two experimental setups at 80 °C (n = 7).

	RT RSD (%)						
Peak Number	1	2	3	4	5	6	7
With TED	0.236	0.249	0.209	0.199	0.118	0.120	0.100
Without TED	0.347	0.300	0.200	0.226	0.095	0.070	0.062

The RSD values are low overall, which shows the precision of the system. A closer look at the data in Table 2 reveals that the precision of results with and without a TED are comparable.

To analyze for differences in resolution, a closer look at the chromatograms is needed. Figure 2 shows three zoomed-in sections of the chromatogram. In Figure 2A, both peaks are baseline separated. However, with a TED, a higher resolution between the peaks is gained. Figure 2B also shows higher resolution with a TED installed. The depicted peaks show partial separation with a TED installed, whereas without a TED, only one peak was observed. Figure 2C shows almost baseline separated peaks with a TED compared to only partial resolution without a TED. These three examples prove that resolution increases significantly with the installation of the TEDs.

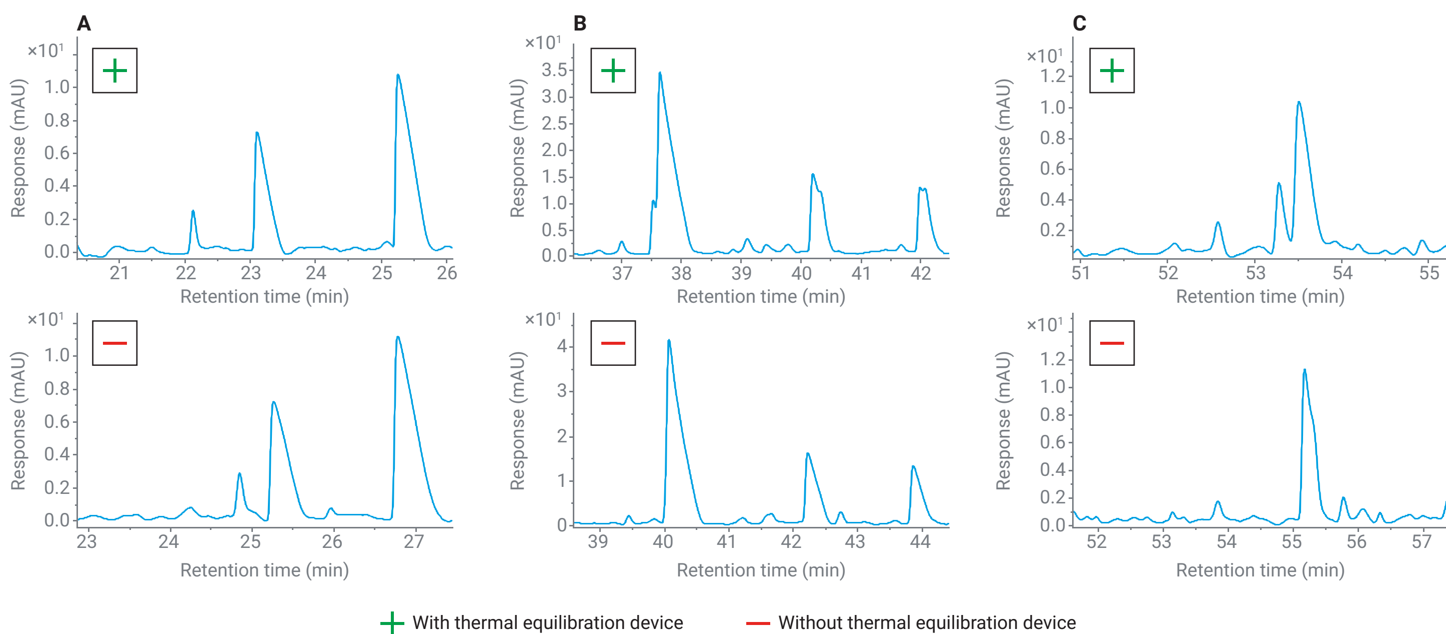

Figure 2. Three zoomed-in sections of the chromatogram illustrating the improvement in resolution using an Agilent Thermal Equilibration Device (TED) for peptide mapping at 80 °C. (A) shows the increased resolution between the two separated peaks with a TED installed in comparison to the resolution without a TED installed. (B) shows partial separation of two peaks with a TED, whereas without a TED, only one peak was observed. (C) shows almost baseline separated peaks with a TED, while without a TED, there is only one peak.

These experiments were also conducted at 40 and 60 °C (see Figure 3). Similar effects could be observed, but the resolution difference becomes less obvious with decreasing temperature.

Overall, at 80 °C, the highest impact on resolution was observed with the TEDs installed.

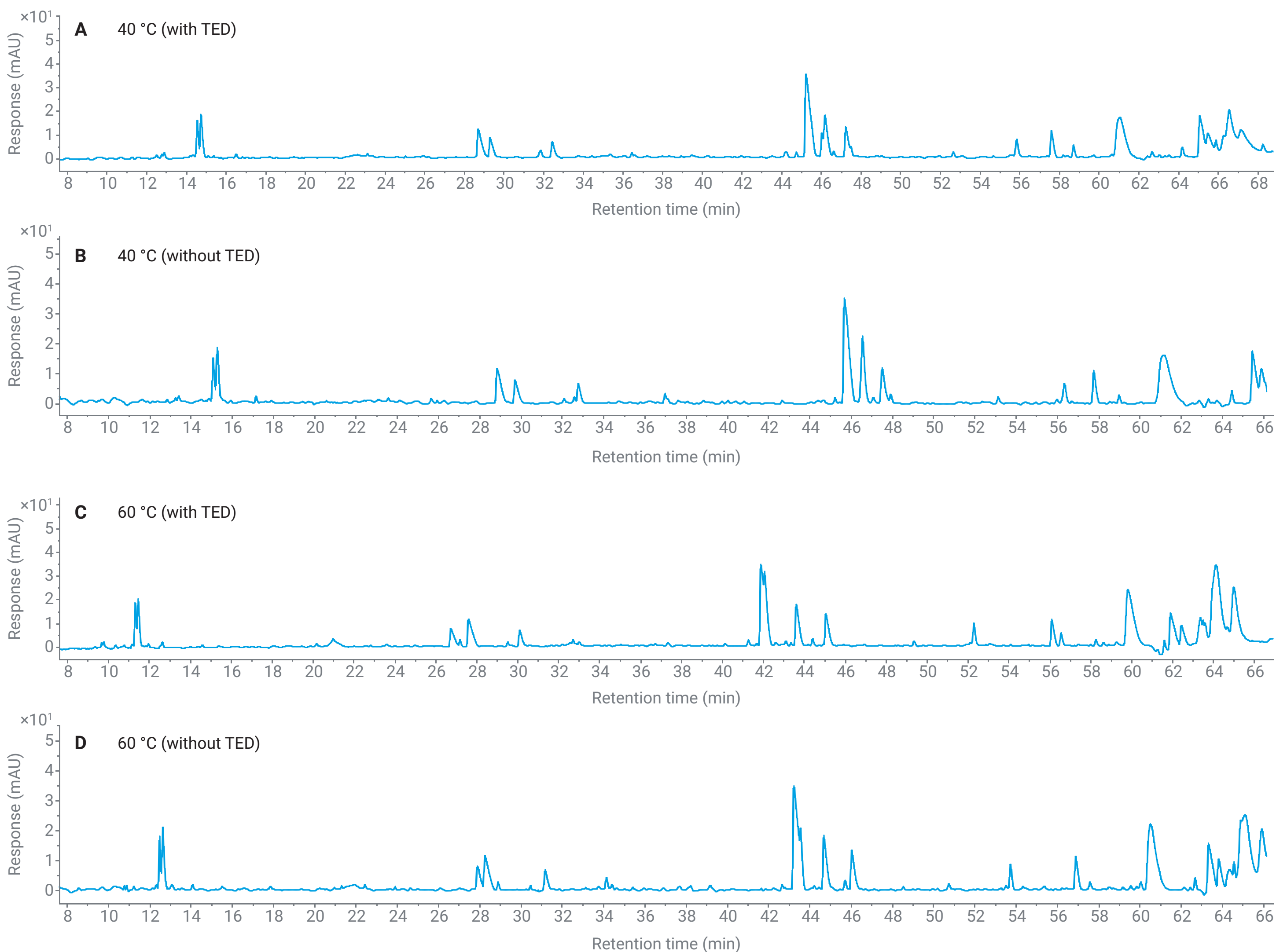

Figure 3. Comparison of peptide-mapping chromatograms with different experimental approaches. A and B show peptide-mapping separations at 40 °C, where a TED was installed only in A. In C and D, peptide mapping was carried out at 60 °C, where a TED was installed in C and not in D.

Conclusion

The Agilent 1290 Infinity II Bio LC with the Agilent Thermal Equilibration Device has been shown to be an ideal combination to improve resolution for peptide mapping at elevated temperatures. The system can therefore be highly recommended.

References

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