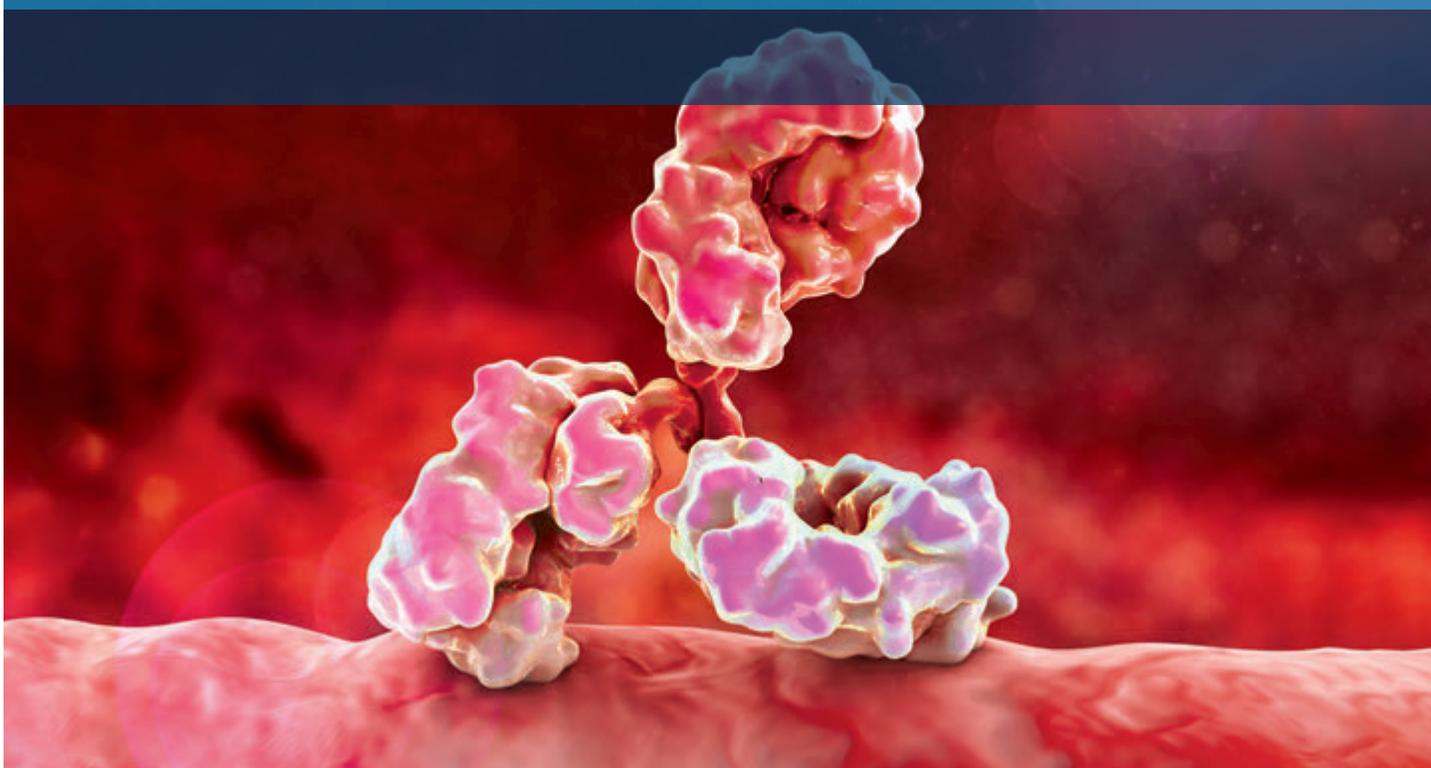


Agilent InfinityLab Bio LC Solutions

# High-Performance Biopharma Analysis

Application compendium



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Trusted Answers



# Overcome Analytical Challenges in Biopharma

The growing complexity of biopharma analysis calls for innovation that delivers excellence across the biopharmaceutical workflow. The Agilent InfinityLab Bio LC Solutions comprise a full range of entirely biocompatible and bio-inert LC systems that enables robust, accurate bioanalysis from drug discovery and development to QA/QC. By combining these systems with bio columns, MS detection, software, and services, we can help you create a complete solution to meet your biopharma challenges.

This application compendium comprises a list of current applications demonstrating the high performance and versatility of the InfinityLab Bio LC Solutions for bioanalysis.

**BIO**



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# Method and System Overview

Use this table as a quick guide to the experimental setup of each application.

	Method						1290 Bio			1260 Bio	
	IEX	SEC	RPLC	HILIC	HIC	AC	HSP	2D-LC	FP	Prime	Inert
<b>Monoclonal Antibodies (mAb)</b>											
Refinement of mAb Charge Variant Analysis	●						●				
Elevated mAb Aggregate Analysis		●					●				
Monitoring of Product-Related mAb Fragments			●				●				
Convenient Customization of Your Cation Exchange Analysis	●									●	
Fully Automated Characterization of Monoclonal Antibody Charge Variants Using 4D-LC/MS	●		●					●			
Two-In-One Bioprocess Analytics				●		●					●
<b>Peptides</b>											
Seamless Method Transfer to the Agilent 1290 Infinity II Bio LC			●				●				
Robust and Reliable Peptide Mapping			●				●				
<b>Antibody-Drug Conjugates (ADCs)</b>											
Analysis of Antibody Drug Conjugates in High-Salt Conditions					●		●				
Convenient and Reliable Analysis of Antibody Drug Conjugates					●					●	
<b>Glycans</b>											
Resolution and Speed in the Separation of Glycans				●			●				
<b>Precursor Oligonucleotides</b>											
Analysis of Raw Material for Oligonucleotide Synthesis			●						●		
<b>Traditional Chinese Medicine</b>											
Performance Comparison to Determine LC Method Compatibility			●							●	

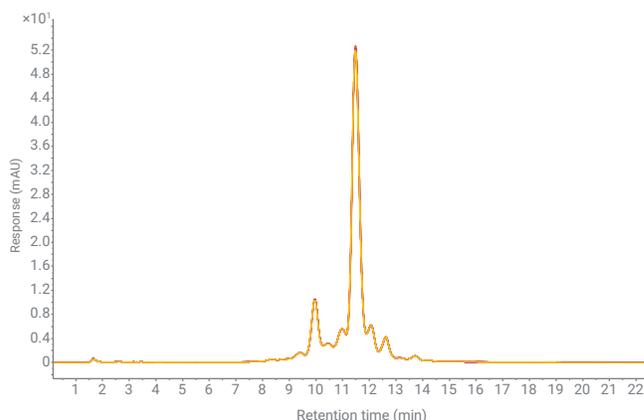
**HSP** Agilent 1290 Infinity II Bio LC with High-Speed Pump  
**2D-LC** Agilent 1290 Infinity II 2D-LC System with Agilent 6545XT AdvanceBio LC/Q-TOF

**FP** Agilent 1290 Infinity II Bio LC with Flexible Pump  
**Prime** Agilent 1260 Infinity II Prime Bio LC  
**Inert** Agilent 1260 Infinity II Bio-Inert LC



## How Shallow Can You Go?

### Refining charge variant analysis of mAbs with the Agilent 1290 Infinity II Bio LC System

**BIO**

#### Author

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#### Abstract

Charge variant analysis is a demanding application for applied liquid chromatography systems due to the use of highly corrosive buffer salts in combination with very shallow gradients for optimal separation. The evaluation of different salt gradients was performed on the Agilent 1290 Infinity II Bio LC System and analyzed for resolution as well as reproducibility.

The 1290 Infinity II Bio LC including High-Speed Pump, with its completely iron-free flow path, is optimally suited for the conditions used in biochromatography—avoiding potentially corrosive damage to the system. Excellent reproducibility even for highly challenging shallow gradients was determined, confirming the 1290 Infinity II Bio LC as the next generation of Agilent high-end liquid chromatography systems for high confidence in generated data.

## Introduction

Monoclonal antibodies (mAbs) are large and highly heterogeneous macromolecules, with a size of around 150 kDa, that are typically generated by recombinant production methods. They are generated in a complex biosynthetic process in which plenty of modifications can occur, leading to hundreds of different variants. Deamidation, oxidation, disulfide bridges, N-glycosylation, N- and C-terminal processing are some of the most common post-translational modifications (PTMs). All these modifications can occur during generation, but also manufacturing and storage contribute to the complexity of these macromolecules. PTMs form a complex isoform profile that needs to be extensively analyzed and monitored, as modifications in the final pharmaceutical might be associated with a loss of biological activity, affected half-life, or immunogenicity.<sup>1</sup> Some of the PTMs result in charge variants of the molecule, which are typically analyzed using ion-exchange chromatography (IEX).<sup>2</sup> Charge variants are considered one of the most important critical quality attributes (CQAs) and therefore strict acceptance criteria and quality controls are to be considered. It is of utmost importance to confirm that the product is correctly manufactured, and to identify and quantify any impurities.

Shallow gradient elution is very common in IEX of proteins. A typical salt gradient in ionic strength mode for the elution of proteins would be approximately 1 to 3 mM/min with a pH value set to a tolerance of  $\pm 0.02$  pH units.<sup>3</sup>

The 1290 Infinity II Bio LC is equipped with a high-performance High-Speed Pump. The major advantage of binary pumps is that solvent mixing is much more accurate and precise when mixing small proportions of one of the solvent components compared to low-pressure mixing pumps (e.g., quaternary pumps). This type of mixing gives highly precise solvent compositions at the start and the end of a solvent gradient.<sup>4</sup> This is a basis for the generation of reproducible and accurate shallow gradients (below 1%/min from each channel).

The 1290 Infinity II Bio LC is the next generation of Agilent high-end liquid chromatography systems, specially designed for conditions used in biochromatography: high salt concentrations such as 2 M NaCl, up to 8 M urea, and high- and low-pH solvents such as 0.5 M NaOH or 0.5 M HCl. The complete flow path is completely free of stainless steel (SST) or iron; all capillaries and fittings throughout the multisampler, multicolumn thermostat, and detectors are built of MP35N, a nickel-cobalt alloy. With this material, potential corrosion from high salt-containing buffers is reduced and protein modifications caused by the presence of iron ions (e.g. oxidation, protein complex formation) can be avoided.

This application note presents the analysis of charge variants for trastuzumab and the NISTmAb reference standard. Different salt gradient slopes were tested to find the best resolution possible. The best performing gradient slopes were then evaluated for reproducibility.

## 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) with the standard flow biocompatible heat exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2  $\mu$ L

### Software

Agilent OpenLab CDS Version 2.5

### Columns

Bio MAb, NP5, 2.1  $\times$  250 mm, PEEK (part number 5190-2411)

### Chemicals

All solvents were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22  $\mu$ m membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, sodium chloride, hydrochloric acid, sodium hydroxide, tris(hydroxymethyl)-aminomethane, imidazole, and piperazine hexahydrate were obtained from Sigma-Aldrich (Steinheim, Germany).

## Samples

- Agilent-NISTmAb (p/n 5191-5744)
- Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland)
- The trastuzumab was dissolved in 30 mM phosphate buffer, pH 6.8

## Buffer preparation

For 2 L of 30 mM phosphate buffer, pH 6.8, 4.45 g of sodium phosphate monobasic monohydrate and 7.44 g of sodium phosphate dibasic heptahydrate was weighed and added to an amber-colored 2 L bottle and filled up to 2 L using ultrapure water (→buffer A). 29.22 g of sodium chloride, for a total concentration of 500 mM, was added to an empty, amber-colored 1 L bottle and filled up to 1 L using the prepared phosphate buffer A (→buffer B). The pH values of both prepared buffers were checked and adjusted, if necessary, to pH 6.8 (the addition of high amounts of salt can change the pH). Both prepared buffers were filtered using a 0.2 µm membrane filter.

## Method

See Table 1.

**Note:** When using concentrated salt solutions as eluents, consider setting corresponding solvent types in the pump method. For example, for Solvent B, including 500 mM sodium chloride, use “Sodium Chloride 0.5 M” rather than *Generic Aqueous* or *Water* in the solvent selection field in the pump method. High amounts of salts change the compressibility of the solvent, and so using the preconfigured solvent tables enables best pump performance.

## Results and discussion

### Method development

To achieve the desired resolution and enable optimal separation, extensive method development is necessary for charge variant analysis. Two parameters are essential to be successful: finding the optimal pH as well as the optimal gradient slope. Both factors can have a major impact on the separation. First, pH scouting is recommended to find the optimal pH for the separation. In earlier experiments, the pH of the used buffers was analyzed from pH 6.4 to 7.4 and was found to be optimal at pH 6.8 for both samples used: trastuzumab and the NISTmAb reference standard (data not shown). The next step is the determination of the ideal gradient slope to enable the efficient separation.

**Table 1.** Salt gradient chromatographic conditions.

Parameter	Value
Solvent	A) 30 mM phosphate buffer, pH 6.8, B: 30 mM phosphate buffer, pH 6.8, 500 mM sodium chloride
Gradient	0 or 25 mM–150 mM NaCl in 30 minutes—different shallow gradients for method development 0 mM (trastuzumab) and 25 mM (NIST) to 100 mM NaCl in 30 minutes for reproducibility 25 to 50 mM NaCl in 30 minutes for reproducibility (very shallow gradient) 31 minutes–500 mM NaCl wash  Stoptime: 35 minutes Post-time: 15 minutes
Flow Rate	0.200 mL/min
Temperature	30 °C
Detection	280 nm 10 Hz
Injection	Injection volume: 3 µL for trastuzumab and 2 µL for NIST Sample temperature: 10 °C Needle wash: 3 s in water

Figure 1 shows an overlay of charge variant analysis of trastuzumab at different gradient slopes ranging from 1% B/min (5 mM/min) down to 0.33% B/min (1.66 mM/min). The shallower the gradient, the higher the requirements to the pump performance. To deliver highly precise solvent compositions during the gradient, the pump needs to work accurately and precisely when mixing small proportions of the solvent components. It has to be considered, though, that for salt gradients, very shallow gradients do not always result in higher resolution, but simply increase peak width (e.g. 0.33% B/min in Figure 1). Therefore, the chosen gradient slope for reproducibility studies was found in the middle of the tested gradients with 0.66% B/min and 3.3 mM/min, which can still be considered shallow.

A similar method development procedure was carried out for the separation of charge variants of the NISTmAb reference standard (see Figure 2). The starting conditions for NIST contained a slightly higher amount of salt due to the higher isoelectric point (pI) of the NIST antibody (pI of ~9.2), compared to trastuzumab with a pI of ~9. For more effective separation of the charge variants of the NISTmAb reference standard, the gradients were slightly shallower compared to trastuzumab, so the most shallow gradient was at 0.17% B/min (0.83 mM/min), which is a challenging task for the pump. For further reproducibility studies, the 0.5% B/min (2.5 mM/min) gradient slope was chosen.

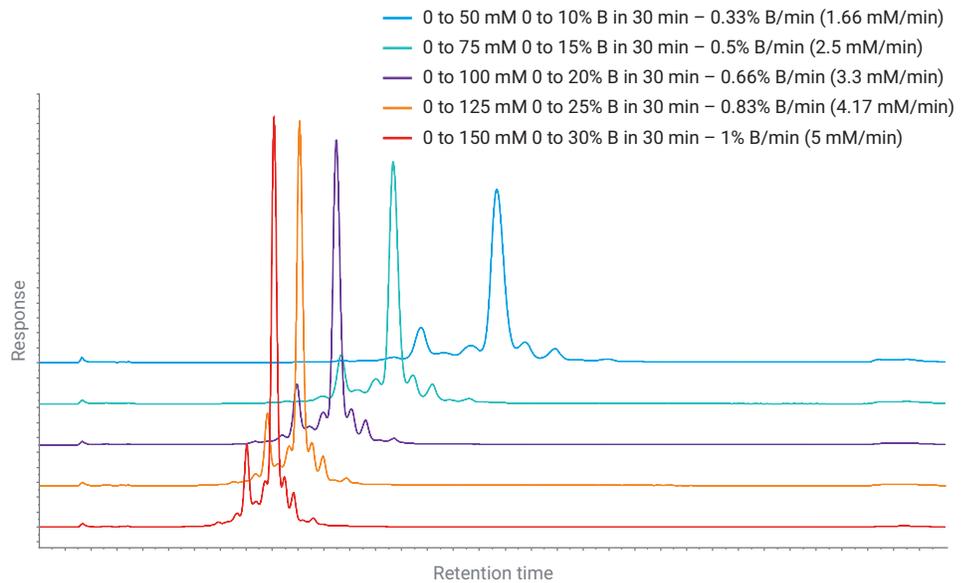


Figure 1. Method development for the separation of trastuzumab with different salt gradient slopes.

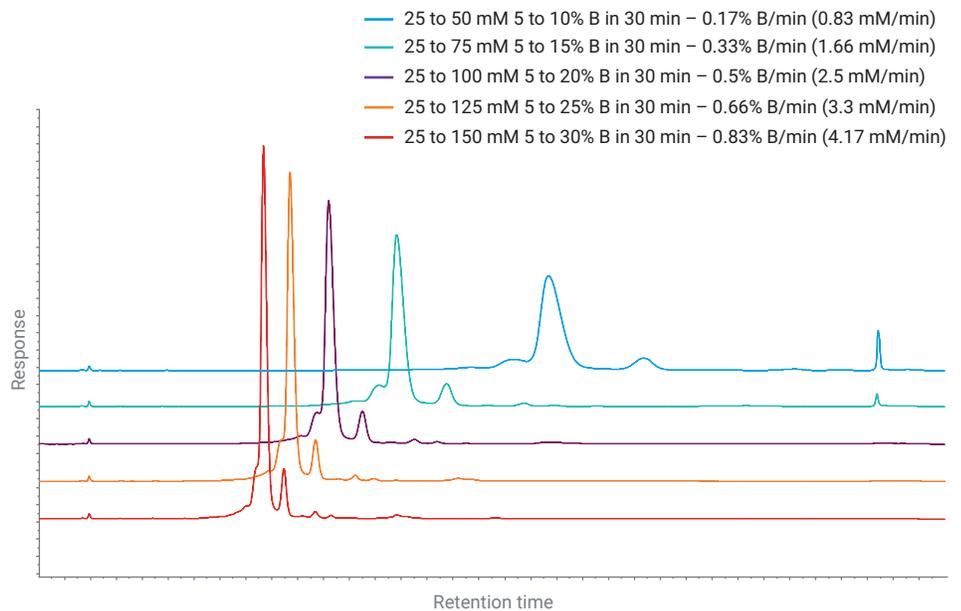
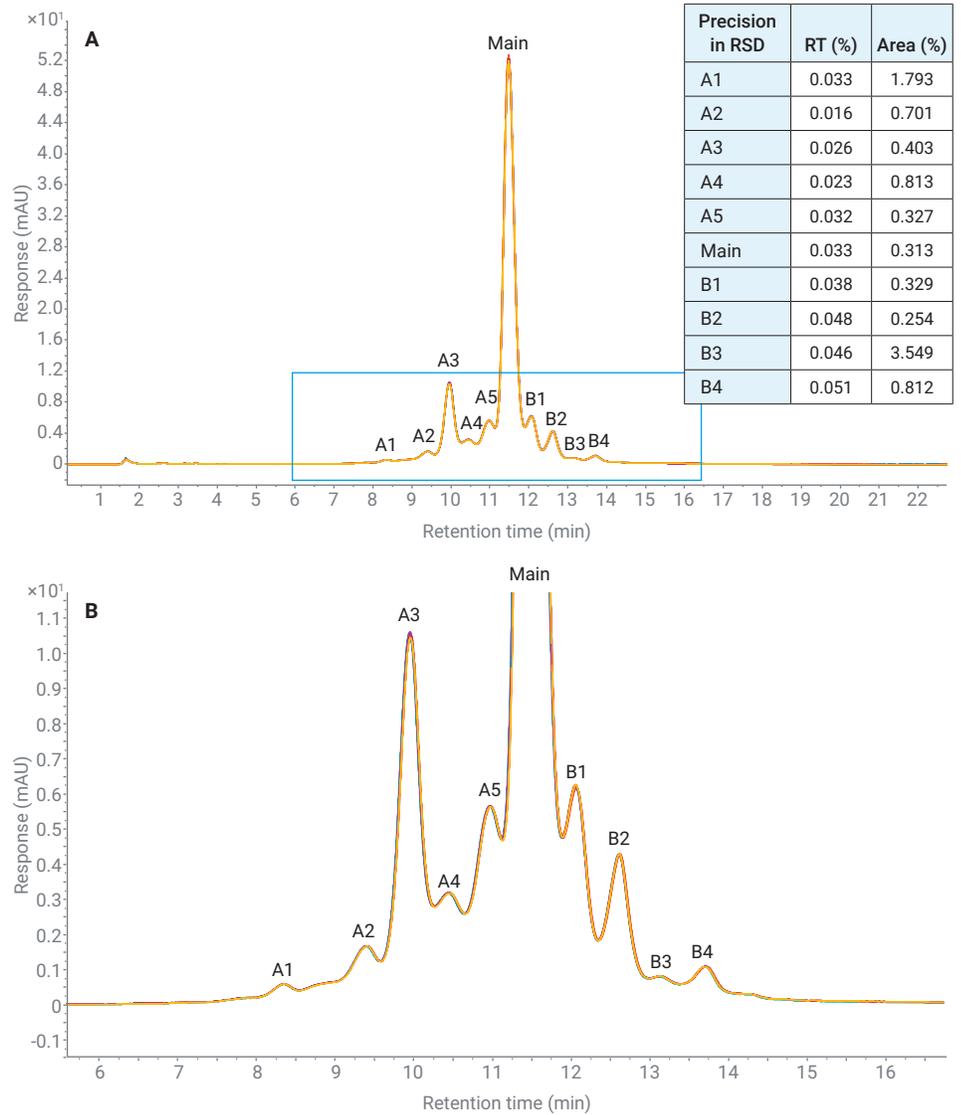


Figure 2. Method development for the separation of the NISTmAb reference standard with different salt gradient slopes.

### Reproducibility for trastuzumab charge variant separation

Figure 3 displays reproducibility studies for charge variant separation of trastuzumab (A) with a 0.66% B/min (3.3 mM/min) gradient slope. Figure 3B shows a zoomed view for better visualization of the separated variants. Variants marked with A represent the acidic variants eluting before the main peaks, whereas the basic variants B elute after the main peak. Five acidic variants were resolved before the main peaks and four basic variants eluted after the main peak. All variants and the main peak were evaluated for the precision of retention time (RT) and area. Both RT as well as area precision are excellent, with values below 0.052% relative standard deviation (RSD) for RT and below 0.82% RSD for area except for the two very small variant peaks A1 and B3.



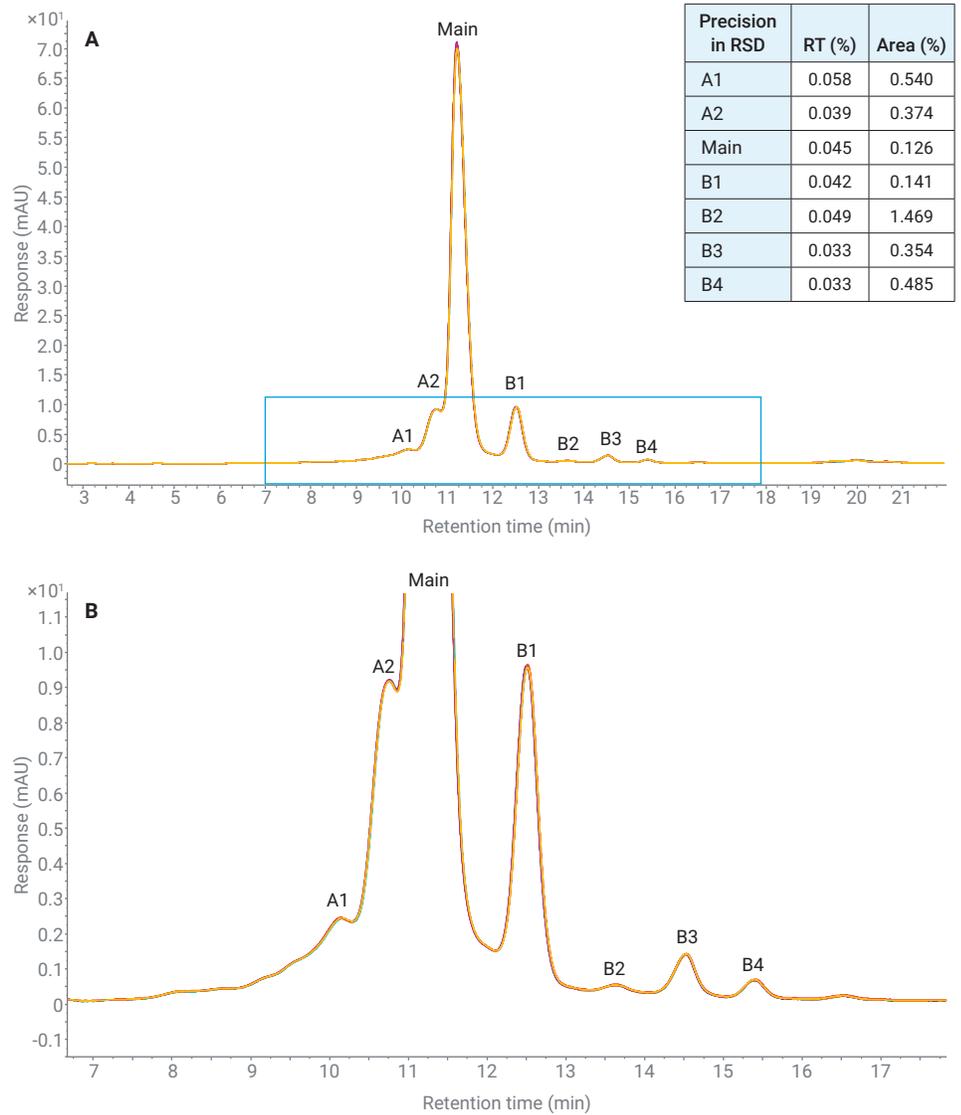
**Figure 3.** Reproducibility studies with seven subsequent runs for charge variant separation of trastuzumab (A) with 0.66% B/min (3.3 mM/min) gradient slope. (B) Zoomed view.

### Reproducibility for NISTmAb charge variant separation

Figure 4 displays reproducibility studies for charge variant separation of the NISTmAb reference standard (A) with 0.5% B/min (2.5 mM/min) gradient slope.

Figure 4B displays the zoomed view with two acidic variants and four basic variants. Again, all variants and the main peak were evaluated for precision of retention time (RT) and area. Both RT as well as area precision are excellent, with values below 0.06% RSD for RT and below 0.55% RSD for area except for one very small variant peak, B2.

As shown in Figure 2, the shallowest gradient with 0.17% B/min (0.83 mM/min) does not deliver a better resolution compared to gradients such as 0.5% B/min (2.5 mM/min) (still shallow—used in the reproducibility studies).

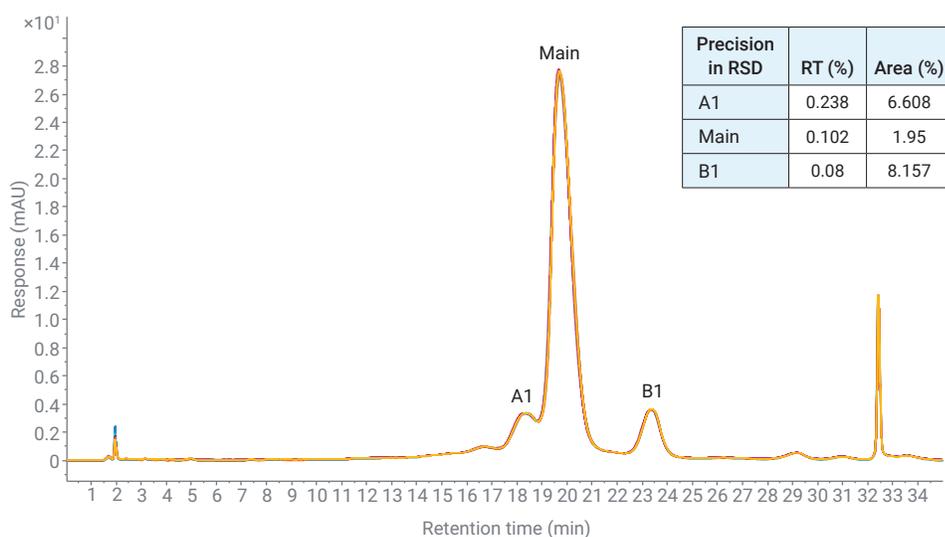


**Figure 4.** Reproducibility studies with seven subsequent runs for charge variant separation of the NISTmAb reference standard (A) with 0.5% B/min (2.5 mM/min) gradient slope. (B) Zoomed view.

However, the 1290 Infinity II Bio High-Speed Pump managed the challenging gradient slope, which is displayed in Figure 5. RT precision over seven subsequent runs was very good (below 0.25% RSD), although the peaks became quite broad with the applied gradient. With increasing peak width, the peak height decreases, which negatively affects the area precision.

## Conclusion

Different salt gradient slopes were evaluated for resolution and reproducibility of the separation of charge variants for trastuzumab and NISTmAb on the 1290 Infinity II Bio LC. At first glance, shallower gradients seemed to improve resolution. However, for both mAbs, the evaluated most shallow gradients did not give the best resolution, as with decreasing slope, the peaks only began to broaden, which led to no further improvement of resolution. The methods with the best combination of high resolution and sharp peak shapes were further evaluated for reproducibility. For a gradient slope of 3.3 mM/min (trastuzumab) and 2.5 mM/min (NISTmAb), excellent reproducibility for RT but also area was documented. The RT precision was below 0.06% RSD for all evaluated peaks. The most shallow gradient tested for NISTmAb was also evaluated for reproducibility, and even for a super shallow gradient with 0.83 mM/min gradient slope, very good RT precision was found (<0.25% RSD). These data show that the 1290 Infinity II Bio LC with its completely iron-free flow path is optimally suited for the conditions used in biochromatography, leading to highly reproducible results.



**Figure 5.** Reproducibility studies with seven subsequent runs for charge variant separation of the NISTmAb reference standard with 0.17% B/min (0.83 mM/min) gradient slope.

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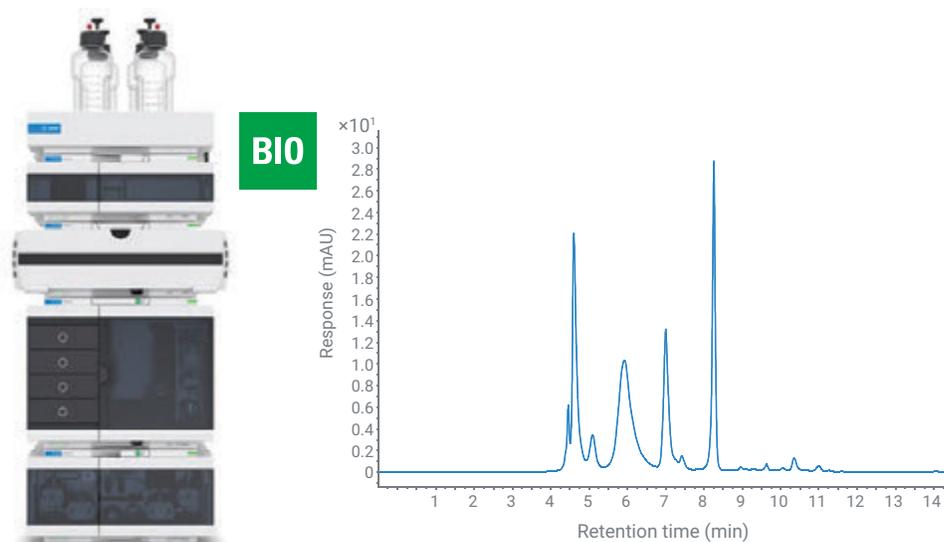
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# Elevate Your mAb Aggregate Analysis

High-resolution SEC with the Agilent 1290 Infinity II Bio LC System



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## Abstract

This application note demonstrates the superior resolution in size exclusion chromatography (SEC) protein separation made possible by the Agilent 1290 Infinity II Bio LC System equipped with the Agilent AdvanceBio SEC column and ultralow dispersion capillaries. The biocompatible UHPLC system enables analysis using corrosive salty buffers and therefore saves maintenance expense. The resolution in SEC analysis was compared for capillaries of different inner diameter (0.17, 0.12, and 0.07 mm). A protein standard mixture and monoclonal antibodies (mAbs), including aggregates, were separated and compared for resolution. In addition, molecular weight was determined by the Agilent OpenLab GPC/SEC add-on software in one software solution, enabling a one-step workflow.

## Introduction

Modern biopharmaceuticals, such as mAbs, are highly heterogeneous compounds. Aggregation monitoring, one of the most important critical quality attributes (CQAs), is typically executed by SEC. With this technique, the identity of the compound can be determined by the calculation of the molecular weight after a standard column calibration. In addition, it confirms the purity by showing the presence of unwanted higher molecular weight compounds such as dimeric and higher aggregates. To achieve the necessary resolution, modern SEC columns with sub-2  $\mu\text{m}$  particle material are recommended. To enable optimal performance, a combination of sub-2  $\mu\text{m}$  columns and a UHPLC instrument with dead volumes as low as possible is preferred. Large dead volumes destroy the resolution obtained by these columns due to dispersion effects. In addition, the completely biocompatible 1290 Infinity II Bio LC perfectly copes with the high salt concentrations often found in SEC buffers, providing confident results at the lowest maintenance cost.

This application note demonstrates the use of modern sub-2  $\mu\text{m}$  SEC columns on the 1290 Infinity II Bio LC and illustrates the benefit of using instruments with the lowest possible dead volumes. To demonstrate the effect of dead volume on the separation of proteins and aggregates, capillaries with different inner diameters were used. The well-characterized NISTmAb will be used to generate more aggregates by pH and thermal stress with subsequent separation of dimers, trimers, and higher aggregates.

## Experimental

### Instrument

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) including integrated Sample Thermostat (#101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with biocompatible Heat Exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2  $\mu\text{L}$

### Additional parts

Agilent 1290 Infinity II Bio Ultra Low Dispersion Kit (G7132A#006)

### Software

Agilent OpenLab Version 2.5 and GPC/SEC add-on software V. 1.2

### LC method

Parameter	Value
Solvent	Phosphate-buffered saline (PBS), pH 7.4
Flow Rate	0.35 mL/min
Isocratic Separation	
Column Temperature	30 °C
Sample Temperature	4 °C
Needle Wash	3 s water
Injection Volume	5 $\mu\text{L}$
Detection (VWD)	280 nm, data rate 20 Hz

### Column

Agilent AdvanceBio SEC, 200  $\text{\AA}$ , 4.6  $\times$  300 mm, 1.9  $\mu\text{m}$  (part number PL1580-5201)

### Samples

- Protein mix for calibration (part number 5190-9417): thyroglobulin (670,000 Da),  $\gamma$ -globulin (150,000 Da), ovalbumin (45,000 Da), myoglobin (17,000 Da), angiotensin II (1,000 Da)
- Humanized monoclonal antibody (mAb) trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland). The trastuzumab was dissolved in 30 mM phosphate buffer, pH 6.8.
- Agilent NISTmAb, humanized IgG1 $\kappa$  mAb (part number 5191-5745)

### Protocol for pH/temperature-stressed NISTmAb

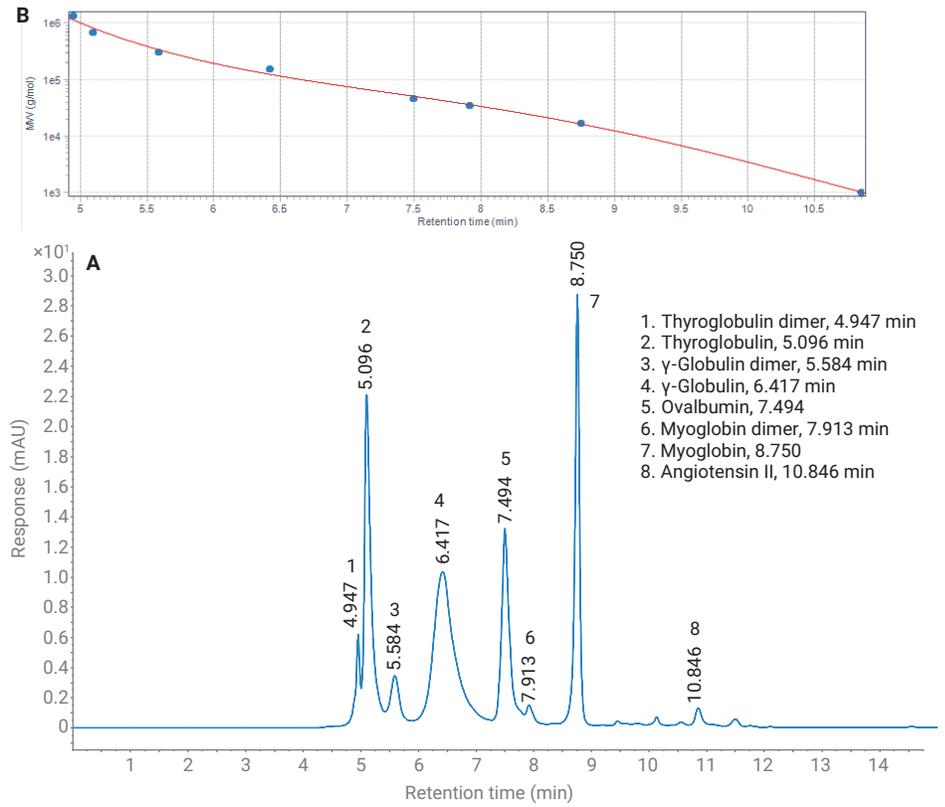
The mAb was diluted in the mobile phase to a final concentration of 2 mg/mL. pH stress was carried out as described elsewhere with slight modification:<sup>1</sup> 1 M HCl was slowly added dropwise to the sample solutions to change the pH from 6.0 to 1.0. Then, 1 M NaOH was added to adjust the pH to 10.0. Finally, 1 M HCl was added again to adjust the pH back to 6.0. There was approximately 1 minute waiting time between the pH shifts, with constant, slight stirring. The resulting solution was incubated at 60 °C for 60 minutes.

### Solvents and chemicals

- **PBS:** One tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride, pH 7.4, at 25 °C.
- Chemicals were purchased from VWR, Germany.
- Fresh ultrapure water was obtained from a Milli-Q integral system equipped with LC-Pak polisher and a 0.22  $\mu\text{m}$  membrane point-of-use cartridge (Millipak).

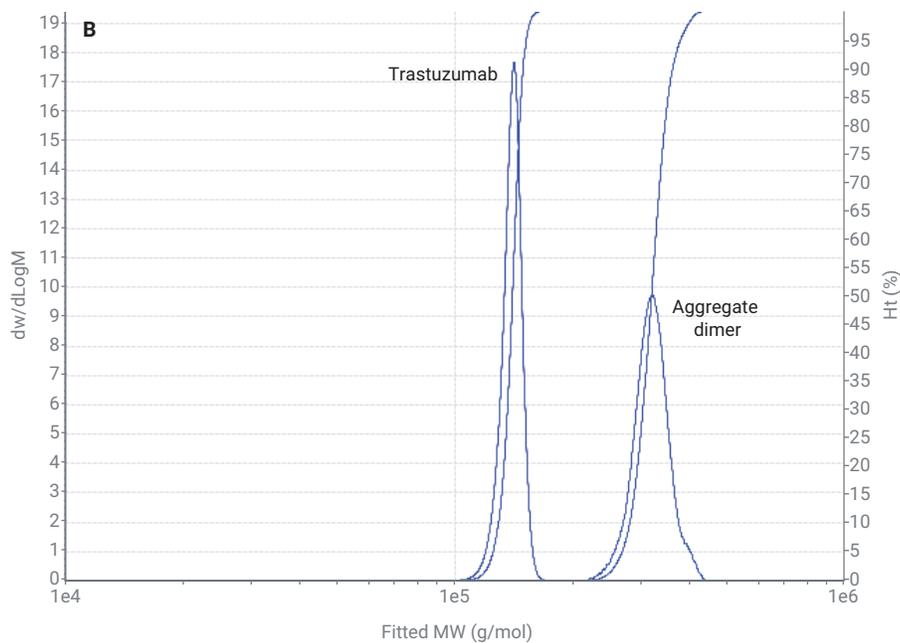
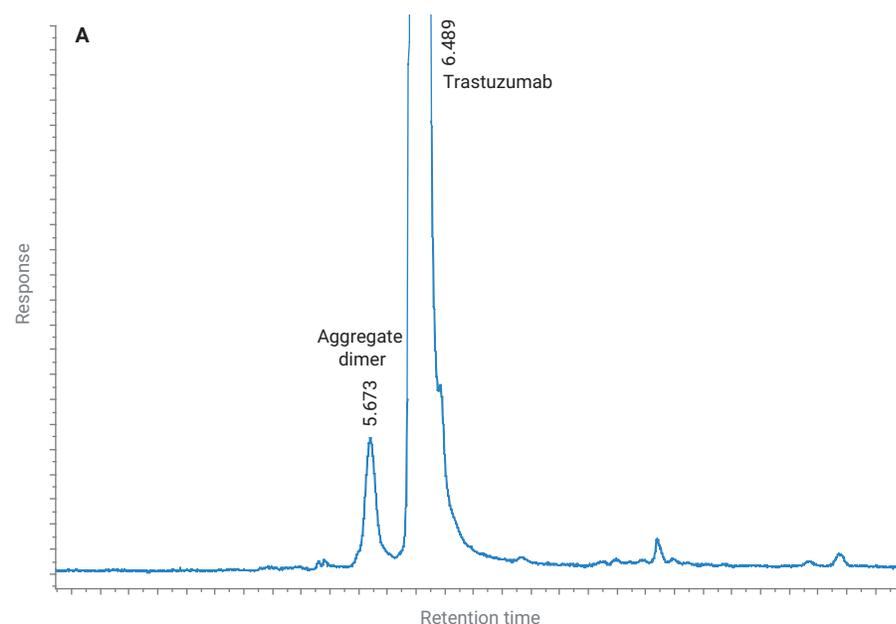
## Results and discussion

Modern columns for SEC separation of proteins comprise material with sub-2  $\mu\text{m}$  particles for optimum resolution. However, this requires instruments that have optimized low dead volume, because especially capillaries of larger inner diameters can destroy the achieved resolution. The separation of a mixture of five proteins, including three dimers, with the 1290 Infinity II Bio LC is shown in Figure 1A. To minimize dead volume and dispersion effects, capillaries with an inner diameter of 0.07 mm were used for the separation. Even the early-eluting dimer of thyroglobulin (4.947 min) was partially separated. To set up a calibration for molecular weight determination, all the proteins in this mixture were used to generate the calibration curve (Figure 1B). The best curve fit was obtained for a fourth order.



**Figure 1.** (A) Separation of a five-protein mixture including three dimers on the Agilent AdvanceBio SEC, 200  $\text{\AA}$ , 4.6  $\times$  300 mm, 1.9  $\mu\text{m}$  in combination with the Agilent 1290 Infinity II Bio Ultra Low Dispersion Kit comprising 0.07 mm capillaries. (B) Calibration curve of SEC for molecular weight determination with a protein mixture featuring some dimers.

The calibration was used to determine the molecular weight of the mAb trastuzumab and a comprised dimeric aggregate (Figure 2). The antibody elutes at 6.489 minutes and the corresponding dimeric aggregate elutes at 5.673 minutes (Figure 2A). The determined molecular weight at the peak maximum of trastuzumab and the dimer were Mp 141,566 Da and Mp 321,609 Da, respectively. The molecular weight distribution is shown in Figure 2B and the calculated molecular weights are outlined in the included table (2C).

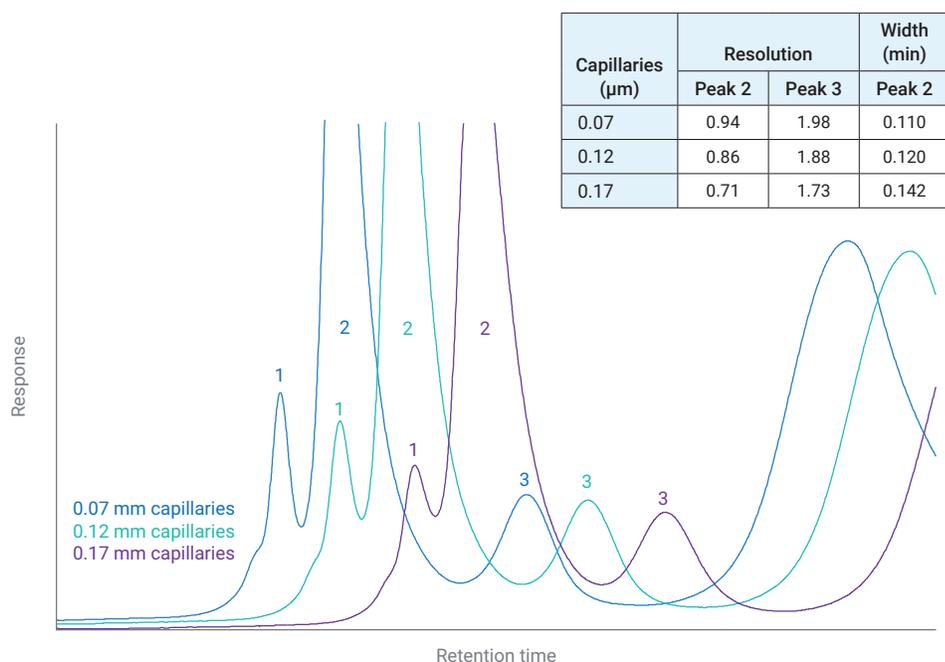


Compound	RT (min)	Mp (g/mol)
Trastuzumab Aggregate	5.673	321609
Trastuzumab	6.489	141566

**Figure 2.** Trastuzumab and dimeric aggregate, determination of molecular weights. (A) SEC separation of the monomer of trastuzumab from a dimeric aggregate. (B) The molecular weight of trastuzumab and the dimeric aggregate. (C) Table with molecular weights of trastuzumab and its aggregate. Mp: molecular weight at peak maximum.

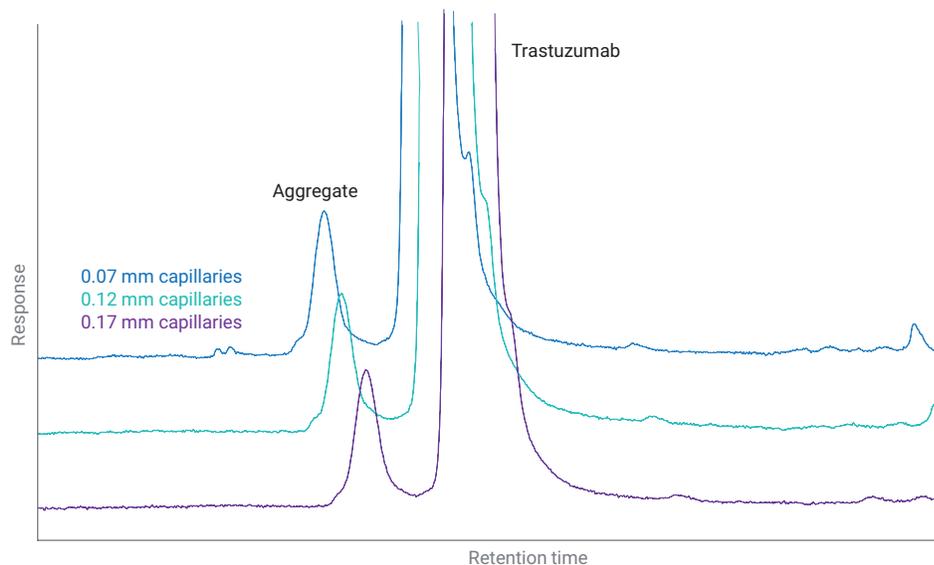
The influence of the inner diameter of the used capillaries could be shown in a comparison of capillaries with an inner diameter of 0.07 (ULD), 0.12 and 0.17 mm. To demonstrate the effect, the resolution of the second and third peak of the protein mixture (shown in Figure 1) was determined (Figure 3). The best values of resolution for peak 2 and peak 3 could be obtained by means of capillaries with 0.07 mm inner diameter (table in Figure 3). For the determination of the peak width at half-height, peak 2 of the protein mixture was used. From the measured values, it could be seen that the peak width increases when using capillaries of larger inner diameters.

The influence of the capillaries on the separation of trastuzumab and its aggregate is shown in Figure 4. Here, it can be seen that an additional lower molecular weight compound was hidden under the main peak, which is only separated as a slight shoulder with the 0.17 mm capillaries and is more clearly visible using the 0.07 mm capillaries.



**Figure 3.** Comparison of capillaries with increasing inner diameters and their influence on resolution and peak width.

Capillaries (µm)	Resolution	Width (min) Herceptin	Width (min) Aggregate
0.07	3.34	0.096	0.189
0.12	3.05	0.107	0.205
0.17	2.83	0.121	0.216



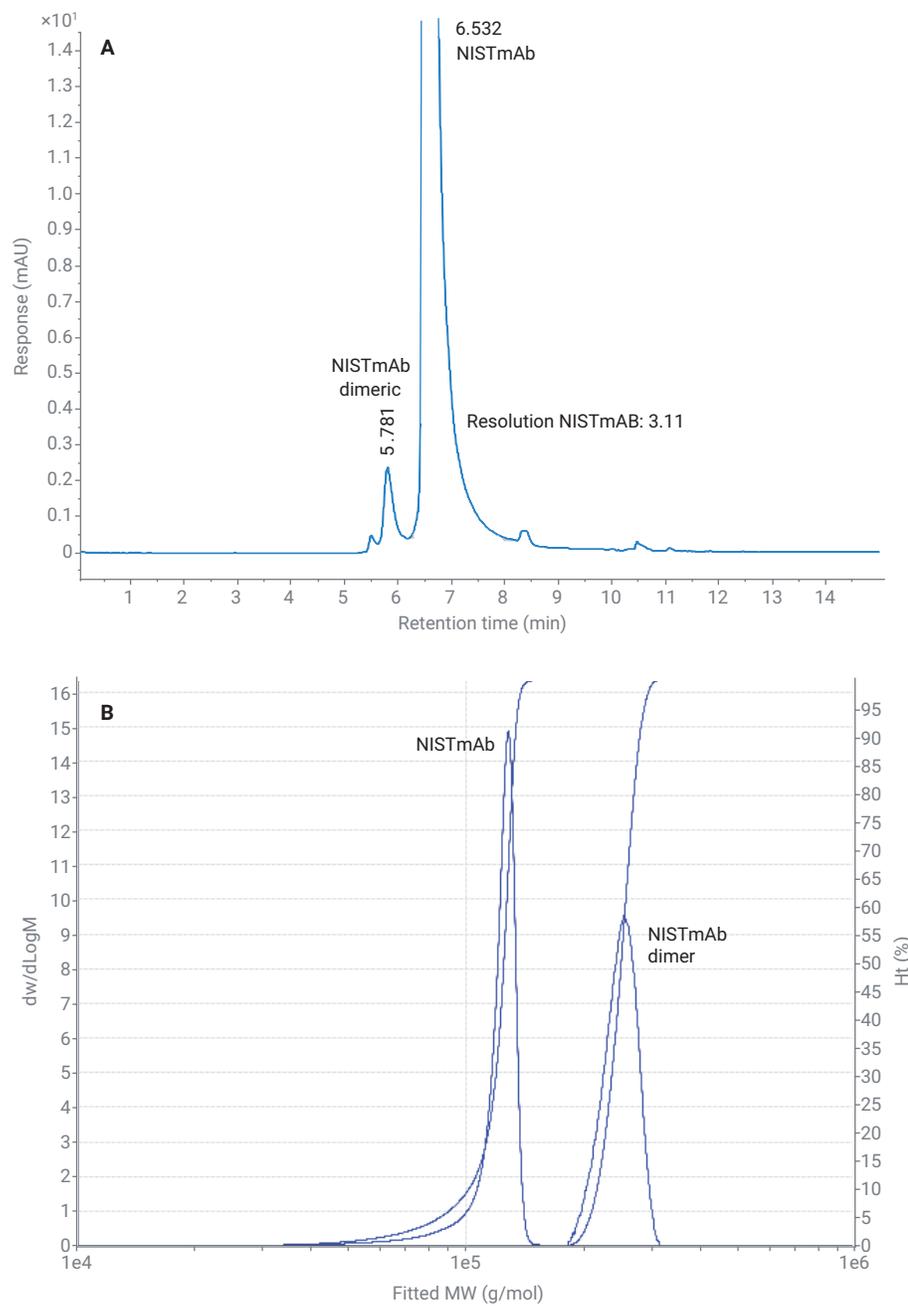
**Figure 4.** Resolution of trastuzumab from its dimeric aggregate and peak width depending on the inner diameter of the used capillaries.

The RSD values of retention time and peak area are excellent for all capillaries (Table 1).

As another example, the well-characterized NISTmAb (humanized IgG1 $\kappa$  mAb) was used for separation from aggregates and determination of molecular weights (Figure 5).

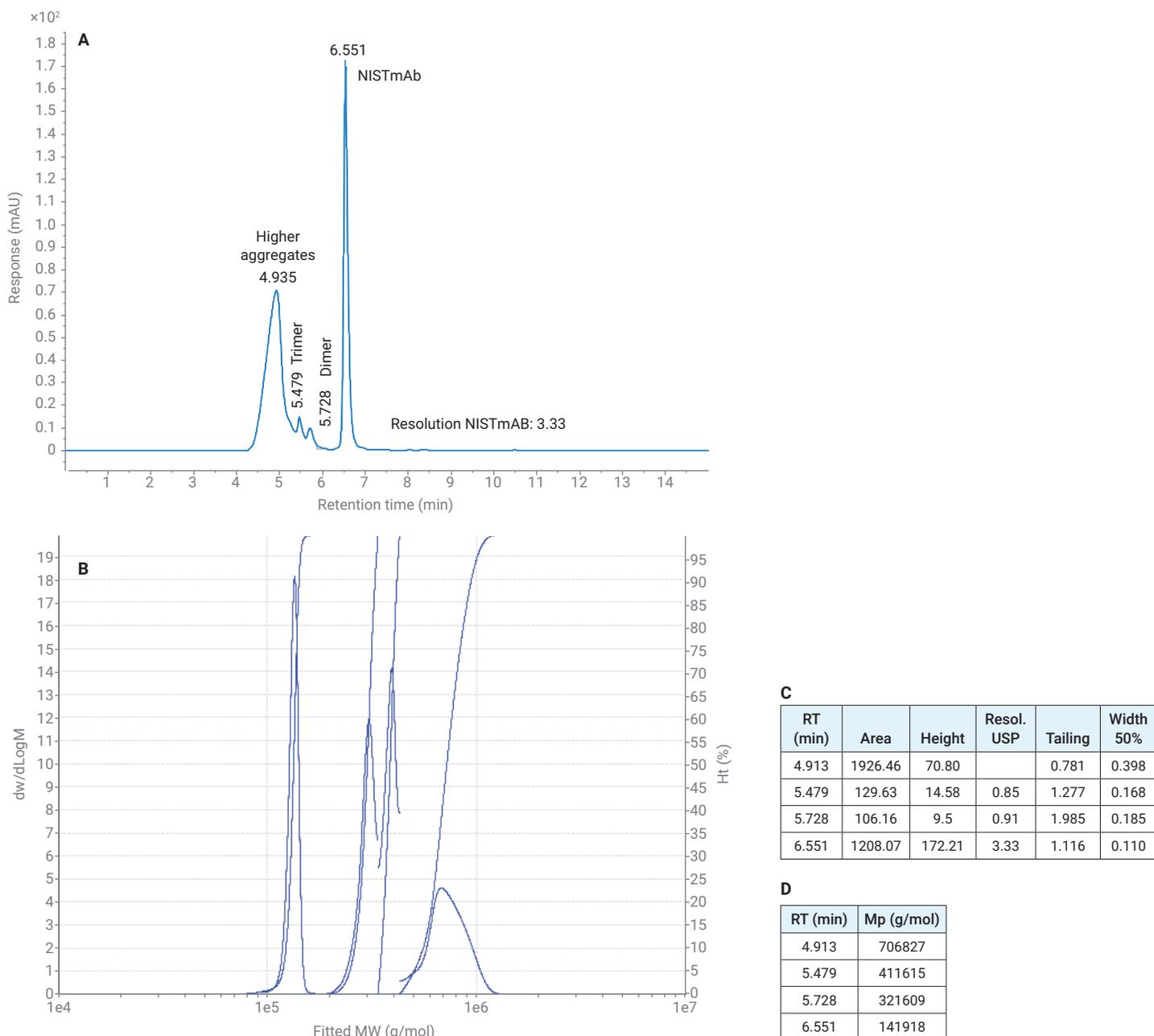
**Table 1.** Retention time and peak area RSDs of trastuzumab for all used capillaries. The increase in retention times is due to the increasing volumes of the different sets of capillaries.

	0.07 mm Capillaries		0.12 mm Capillaries		0.17 mm Capillaries	
	RT	Area	RT	Area	RT	Area
Average	6.464	1736.13	6.500	1727.05	6.554	1717.29
RSD (%)	0.02	0.10	0.01	0.28	0.01	0.25



**Figure 5.** (A) Separation of NISTmAb from its main dimeric aggregate with resolution 3.11. (B) Molecular weights in the NISTmAb and its aggregate. (C) Table showing the values of major peak characterization from NISTmAb and its aggregate. (D) Tables showing the molecular mass of NISTmAb and its aggregate.

Under pH- and temperature-stress conditions (see Experimental section), this mAb can form higher aggregates (Figure 6). With the ultralow dispersion capillaries, the higher aggregates could be separated (Figure 6A). Their molecular weight distribution and values for peak characterization are outlined in Figure 6B and the associated tables.



**Figure 6.** (A) Separation of aggregates from a pH-stressed NISTmAb. (B) Distribution of molecular weight of aggregates occurring under stress conditions from NISTmAb. (C) Table of values of major peak characterization of NISTmAb and its aggregates. (D) Tables showing the molecular mass of NISTmAb and its aggregates.

## Conclusion

This application note demonstrates the capability of the 1290 Infinity II Bio LC together with the AdvanceBio SEC column to separate proteins and their aggregates with the highest resolution due to minimized system dead volume and ultralow dispersion capillaries. The 1290 Infinity II Bio LC is a completely biocompatible system capable of operating with highly salted buffers. This offers the lowest maintenance costs at the highest resolution performance.

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[www.agilent.com/chem](http://www.agilent.com/chem)

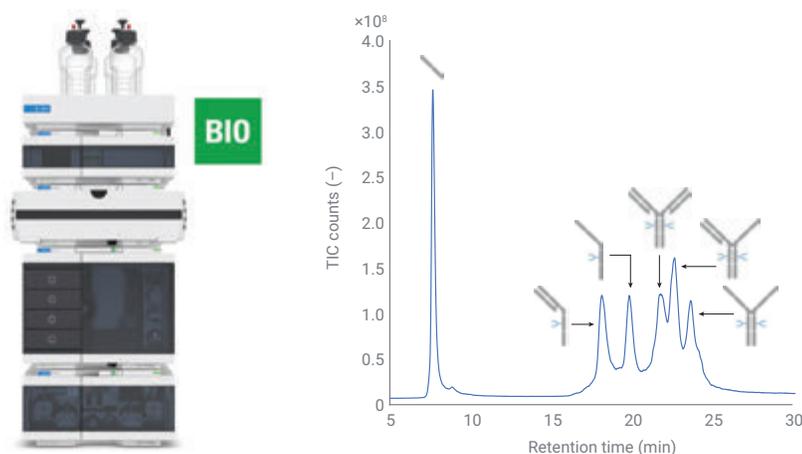
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5994-2709EN

## Monitoring Product-Related mAb Fragments

Intact protein analysis with the Agilent 1290 Infinity II Bio LC System enables UV and MS detection of low molecular weight species



### Author

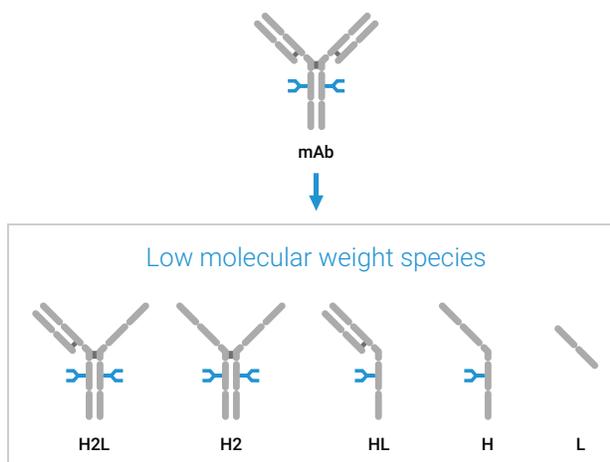
André Feith  
Agilent Technologies, Inc.

### Abstract

Product-related impurities such as low molecular weight (LMW) or high molecular weight (HMW) species are considered critical quality attributes (CQAs) in therapeutic monoclonal antibody (mAb) products and need to be monitored across the drug production process. This application note developed an RPLC method based on the excellent performance of the Agilent 1290 Infinity II Bio LC System combined with the PEEK-lined Agilent PLRP-S column. By analyzing the reduced heavy and light chains of the NISTmAb, excellent relative retention time and area deviations were observed, even with extremely shallow gradient slopes. After method development, all relevant LMW fragments, such as two heavy chains (H2) or two heavy chains and one light chain (H2L), could be separated and detected. Due to the sequential coupling of the UV and MS detector, this method can be used in several areas of the biopharmaceutical production chain. The method also stands as an alternative to SDS-PAGE/CE-SDS with the possibility to analyze two CQAs – LMW species and post-translational modifications (PTMs) – in one run.

## Introduction

mAbs are a major product class of biopharmaceuticals and have been used successfully to tackle various diseases.<sup>1</sup> These biomolecules possess a conserved heterotetrameric structure, consisting of two heavy chains and two light chains connected by disulfide bonds. During manufacturing or improper storage, product-related impurities such as LMW species (see Figure 1) or HMW species (e.g., antibody dimers) can be formed. Those impurities can be present even after extensive purification steps, making it essential to monitor them as a CQA for a drug product. HMW species such as antibody dimers, trimers, or higher aggregates can routinely be analyzed and separated by size exclusion chromatography (SEC) with UV detection.<sup>2</sup> Coupling of SEC with MS detection can be performed to further characterize impurities regarding molecular weight and PTMs.<sup>3</sup> The analysis of LMW species such as heavy chain (H), light chain (L), or H2L fragments can be carried out by capillary electrophoresis-sodium dodecyl sulfate (CE-SDS).<sup>4</sup> Unfortunately, CE-SDS cannot be coupled to MS detection due to high ion suppression caused by SDS, and therefore proposed identities of LMW species are often based on empirical knowledge. This application note shows an alternative analysis of LMW species of mAbs based on the excellent performance of the 1290 Infinity II Bio LC and the PEEK-lined PLRP-S column. Due to the reversed-phase liquid chromatography (RPLC) mode, all relevant reduction-induced LMW fragments of the NISTmAb can be detected with UV and MS for routine or in-depth analysis as needed.



**Figure 1.** Schematic overview of reduction-induced LMW species of monoclonal antibodies (mAb). Abbreviations: H2L (two heavy chains and one light chain), H2 (two heavy chains), HL (one heavy chain and one light chain), H (heavy chain), and L (light chain).

## 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 (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  $\mu$ L, RFID.
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6545XT)

### Software

- Agilent MassHunter workstation data acquisition (B.09.00 or later)
- Agilent MassHunter Qualitative Analysis (10.0 or later)
- Agilent MassHunter BioConfirm (10.0 or later)

### Columns

Agilent PLRP-S 5  $\mu$ m 1000  $\text{\AA}$ , 2.1  $\times$  100 mm PEEK-lined (part number PL1912-2502PK)

### Chemicals

Agilent InfinityLab Ultrapure LC/MS acetonitrile (part number 5191-4496) and the Agilent-NISTmAb (part number 5191-5744) were used. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22  $\mu$ m membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). DL-dithiothreitol (DTT) was purchased from Merck (Darmstadt, Germany).

## Sample preparation

To partially reduce the NISTmAb, 40 µg were incubated with 1 mM DTT in an amber glass vial directly in the 1290 Infinity II Bio Multisampler at 4 °C. Full reduction into heavy chain (H) and light chain (L) was achieved by incubating 40 µg of NISTmAb with 10 mM DTT at 60 °C for 30 minutes. Injection concentration was 1 mg/mL NISTmAb or reduced NISTmAb.

**Table 1.** LC method for analyzing the intact NISTmAb and corresponding LMW species with the Agilent 1290 Infinity II Bio LC.

Parameter	Value
Column	Agilent PLRP-S 5 µm 1,000 Å, 2.1 × 100 mm PEEK-lined
Solvent	A) Water + 0.1% formic acid B) Acetonitrile + 0.1% formic acid
Gradient	0.00 min – 25% B 9.00 min – 30% B 34.00 min – 38% B 34.01 min – 100% B 36.00 min – 100% B 36.01 min – 25% B 40.00 min – 25% B
Flow Rate	0.400 mL/min
Temperature	60 °C with thermal equilibration devices installed
UV Detection	VWD: 280 nm, 10 Hz/MS: see Table 2
Injection	Injection volume: 0.3 µL Sample temperature: 4 °C Wash: 3 s with water (flush port)

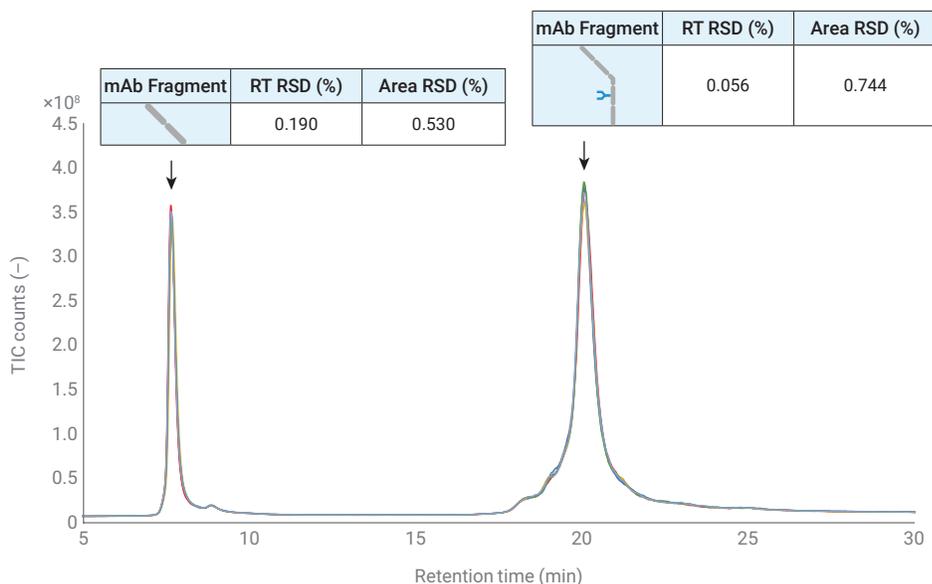
**Table 2.** Source and MS parameters for the analysis of the intact NISTmAb and corresponding LMW species.

Parameter	Value
Instrument	Agilent 6545XT AdvanceBio LC/Q-TOF
Gas Temperature	350 °C
Drying Gas Flow	12 L/min
Nebulizer	35 psig
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
VCap	4,000 V
Nozzle Voltage	2,000 V
Fragmentor	180 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Acquisition Mode	Positive, extended ( <i>m/z</i> 10,000) mass range
Mass Range	<i>m/z</i> 100 to 10,000
Acquisition Rate	1 spectrum/sec
Reference Mass	<i>m/z</i> 922.0098

## Results and discussion

The analysis of biopharmaceuticals throughout the production process from manufacturing to quality control demands the best performance possible from LC systems. To evaluate the 1290 Infinity II Bio LC's performance regarding the analysis of mAb fragments, the NISTmAb was entirely reduced with DTT, resulting in H and L fragments. Figure 2 shows the relative retention time and area standard deviations (RSD) based on seven consecutive injections. It shows that the retention time and area precision of the 1290 Infinity II Bio LC coupled to the 6545XT AdvanceBio LC/Q-TOF is excellent and perfectly suited for analyzing mAb fragments with shallow gradients. Even though the LC method consists of two linear gradient steps with slopes of 0.32 and 0.55 %B/min, the RSD values remain low with 0.190% (L) and 0.056% (H) for the retention time and 0.530% (L) and 0.744% (H) for the area precision.

One of the major challenges when analyzing LMW species with RPLC is the insufficient resolving power to separate antibody fragments such as H2 or H2L due to their similarity in hydrophobicity compared to the actual mAb. These fragments can occur in the fermentation process or by partial reduction in the final product. However, these fragments can also be generated artificially by partial reduction over time with a low amount of DTT and decreased temperature. With this technique, an RPLC method based on the PEEK-lined PLRP-S and the 1290 Infinity II Bio LC was developed.

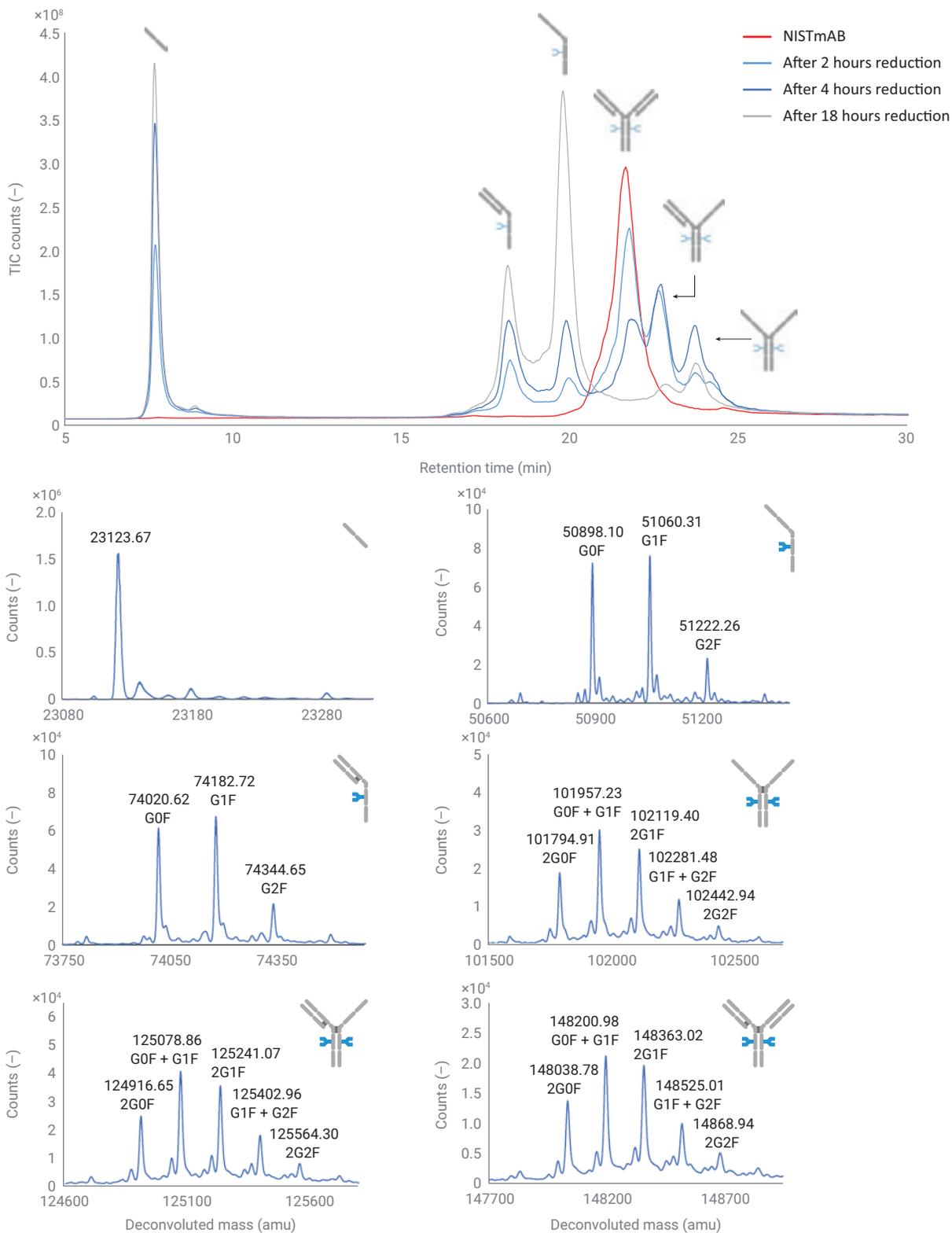


**Figure 2.** Relative retention time and area precision (RSD, n = 7) values for the Agilent 1290 Infinity II Bio LC analyzing heavy and light chain fragments derived by reduction of the NISTmAb.

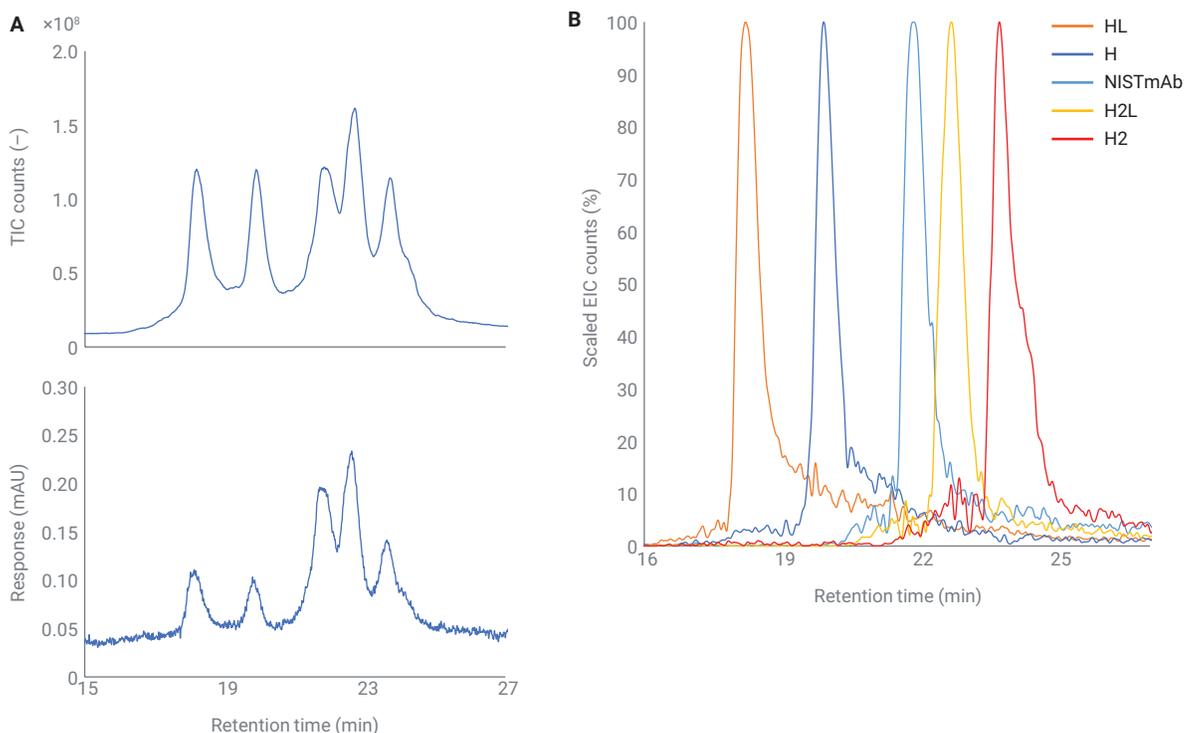
The dynamic reduction of the NISTmAb in the 1290 Infinity II Bio Multisampler can be seen in the chromatogram of Figure 3. All of the relevant mAb fragments depicted in Figure 1 can be nicely resolved and change over time due to the addition of DTT. In particular, the separation of the H2, H2L fragments, and the NISTmAb is exceptionally good for RPLC, rendering the combination of the PEEK-lined PLRP-S column and the 1290 Infinity II Bio LC the method of choice for the analysis of LMW. Thanks to the RPLC mode, the 1290 Infinity II Bio LC System can easily be coupled to the 6545XT AdvanceBio LC/Q-TOF, and MS data can be analyzed in Agilent MassHunter BioConfirm. After deconvolution, the spectra in Figure 3 depict the main glycoforms of the

respective fragments. The characteristic glycosylation of the NISTmAb shows that it is possible to analyze PTMs of the different fragments easily with this method. Additionally, Figure 4B shows the extracted ion chromatograms (EIC) of representative ions for the fragments clustering around the mAb peak. These EICs also offer good peak shape owing to the resolving power of the PEEK-lined PLRP-S column.

Since the instrumentation setup comprises the 1290 Infinity II Variable Wavelength Detector and the 6545XT AdvanceBio LC/Q-TOF in sequence, UV and MS detection is possible in one run with little to no band broadening and convenient method transfer from process development to quality control (Figure 4A).



**Figure 3.** Chromatograms of the dynamic partial reduction of the NISTmAb separated by the Agilent 1290 Infinity II Bio LC and detected with the Agilent 6545XT AdvanceBio LC/Q-TOF. Corresponding extracted spectra of the respective fragments show the characteristic glycosylation of the NISTmAb.



**Figure 4.** MS and UV chromatogram of the fragments clustering around the NISTmAb acquired in one run (A). Extracted ion chromatograms of NISTmAb fragments showing excellent peak shape (B).

## Conclusion

Traditional SDS-PAGE and the modern equivalent CE-SDS are widely used to analyze product-related impurities like LMW and HMW species. However, structural identification of LMW species with these methods has been challenging and primarily based on empirical knowledge. This application note presents an RPLC method capable of separating all relevant reduction-induced LMW species of the NISTmAb. The 1290 Infinity II Bio LC showed excellent retention time and area precision values based on the heavy chain and light chain

fragment analysis. Dynamic reduction of the NISTmAb in the 1290 Infinity II Bio Multisampler and subsequent detection with the 6545XT AdvanceBio LC/Q-TOF showed the potential of the method to analyze post-translational modifications. When combined with fragment analysis, this capability can accelerate biopharmaceutical development. That is why the PEEK-lined PLRP-S column and the 1290 Infinity II Bio LC are a future-proof combination for the analysis of biopharmaceuticals across the production process up to final quality control.

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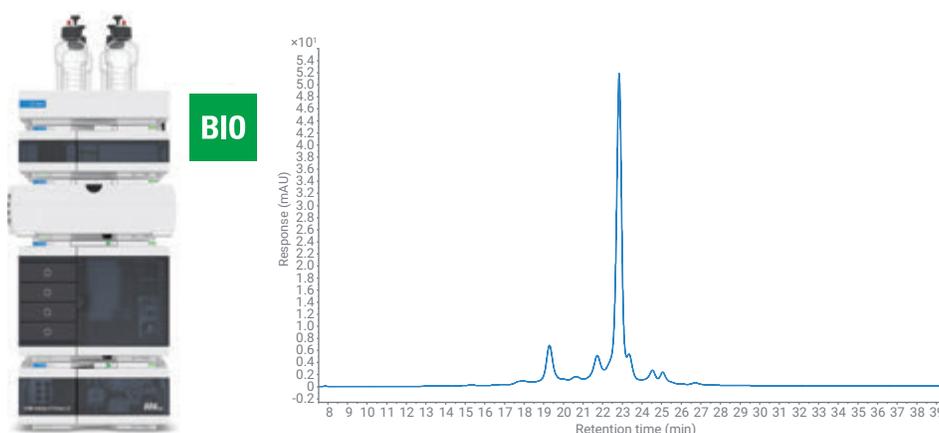
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## Convenient Customization of Your Cation Exchange Analysis

Combining the Agilent 1260 Infinity II Prime Bio LC System, Agilent Buffer Advisor Software, and pH gradients for high-resolving charge variant analysis



### Author

Sonja Schneider  
Agilent Technologies, Inc.

### Abstract

Charge variants separation of monoclonal antibodies can be a challenging task for the chromatographer. Due to the microheterogeneity of the analyzed monoclonal antibody, extensive method development can be necessary to find the optimal desired resolution. Outperforming many traditional salt gradients, the resolving power of pH gradients enables the separation of charge variants in a very efficient way. This application note demonstrates high-resolving and reproducible charge variant analysis of two monoclonal antibodies, trastuzumab and NIST mAb, with different types of pH gradients.

The Agilent 1260 Infinity II Prime Bio LC System, with a completely iron-free flow path and featuring an Agilent 1260 Infinity II Bio Flexible Pump, enables the use of Agilent Buffer Advisor Software to facilitate dynamic mixing of solvents from only four stock solutions.

## Introduction

Therapeutic monoclonal antibodies (mAbs) are highly heterogeneous molecules and are composed of a large number of variants. These are naturally occurring in this kind of biopharmaceutical and are not necessarily considered impurities. Proteins in solution have mostly polar and charged amino acids at the protein interface to aqueous media, while the hydrophobic residues tend to self-associate due to hydrophobic interactions. These amino acids on the “outside” of the proteins that are in contact with surrounding liquid are more predisposed to modifications.<sup>1</sup>

Variants, also called protein microheterogeneity, might originate from post-translational modifications during antibody production. In addition, modifications after purification processes, formulation and/or storage can be formed.<sup>2</sup> However, if the variants are present in the pharmaceutical protein, their biological activity might differ and immunogenicity might be enhanced.<sup>2</sup> Hence, the microheterogeneity of the mAbs is subject to extensive analytical characterization to ensure safety and efficacy of the biopharmaceutical.

Cation exchange chromatography (CEX) is considered the gold standard for charge variant analysis of monoclonal antibodies.<sup>3</sup> Classic salt gradients have high resolving power once the method is fully optimized. However, the amount of effort required to develop a high-resolving ion exchange method for protein separation can be very high. Salt concentration, mobile phase pH values, and additives are only a few of the parameters to be optimized. In addition, every molecule and especially biological molecules might show different behavior and the developed methods are not tolerant to large changes in experimental parameters, especially with respect to pH values.<sup>1,4</sup>

pH gradient-based CEX, also known as chromatofocusing, enables high-resolving as well as robust methods for the separation of mAb charge variants.<sup>1,4,5</sup> In typical ion-exchange chromatography (IEX), the molecules are eluted from the column by increasing the ionic strength (mostly with salts like NaCl) of the buffer. In contrast, with pH gradients, the bound molecules are eluted with the changing pH of the buffer. This alters their net surface charge to enable the elution of the bound molecules at their isoelectric point (pI), where the molecule is electrically neutral.

Wide pH gradient methods are more generic and can separate variants from different antibodies within a single buffer system.<sup>1</sup> Also, the method development of pH gradient-based methods is more straightforward and significantly shorter compared to conventional ionic strength-based IEX.

The 1260 Infinity II Prime Bio LC System is the next generation of Agilent high-end liquid chromatography systems, specially designed for conditions used in bio chromatography: The sample flow path is completely free of stainless steel (SST) or iron; all capillaries and fittings throughout the multisampler, multicolumn thermostat, and detectors are built of MP35N, a nickel-cobalt alloy. With this material, potential corrosion from high salt-containing buffers is reduced and protein modifications caused by the presence of ferric ions (e.g. oxidation and protein complex formation) can be avoided.

The 1260 Infinity II Bio Flexible Pump, as a quaternary pump, enables the use of Buffer Advisor Software to facilitate dynamic mixing of solvents from only four stock solutions, simplifying the bioanalysis workflow and significantly reducing the time required for buffer preparation. With Buffer Advisor Software, quaternary salt gradients as well as pH gradients can be generated

quickly and simply by the calculation of pump timetables for IEX.

This application note presents the analysis of charge variants for trastuzumab and the NIST mAb reference standard with two different pH gradients.

## Experimental

### Equipment

The Agilent 1260 Infinity II Prime Bio LC System comprised the following modules:

- Agilent 1260 Infinity II Bio Flexible Pump (G7131C)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with standard flow bio-compatible heat exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2  $\mu$ L

### Software

Agilent OpenLab CDS version 2.5 or later versions

### Columns

Bio MAb, NP5, 2.1  $\times$  250 mm, PEEK (part number 5190-2411)

### Chemicals

All solvents were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22  $\mu$ m membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, sodium chloride, *tris* (tris(hydroxymethyl) aminomethane), imidazole, hydrochloric acid, and piperazine hexahydrate were obtained from Sigma-Aldrich (Steinheim, Germany).

## Samples

- Agilent-NISTmAb (part number 5191-5744)
- Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland). Trastuzumab was dissolved in 30 mM phosphate buffer, pH 6.8.

## Buffer preparation

The buffers were prepared according to the Stock Solution Recipes from Buffer Advisor Software (see Figure 1).

### Quaternary phosphate-based buffer system—calculated by Buffer Advisor

A: Water

B: 1,700 mM sodium chloride

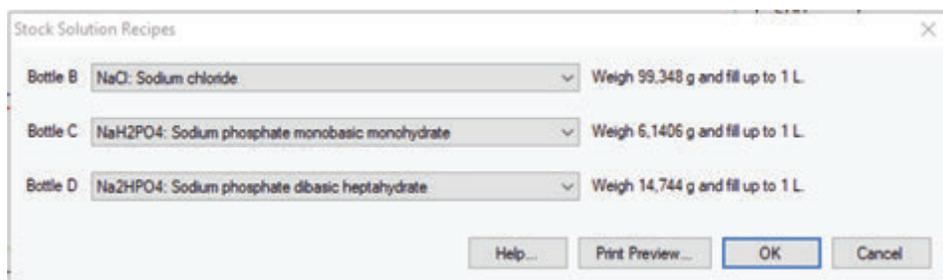
C: 44.5 mM sodium phosphate monobasic

D: 55 mM sodium phosphate dibasic

**Note:** This setup can be used for salt as well as pH gradient elution—enabling direct comparison and increasing the possibilities for method development.

### Binary wide pH gradient buffer system—calculated by Buffer Advisor

With Buffer Advisor, it is also possible to create wide range pH gradients, also termed Composite Buffer. In these cases, only the C and D channels are employed to create the gradient. This experiment used a pH gradient described by Farnan and Moreno<sup>1</sup> and inserted the buffer composition as a User Mixture in the stock solution composition of Buffer Advisor (2.4 mM tris, 1.5 mM imidazole, 11.6 mM piperazine, HCl for pH



**Figure 1.** Agilent Buffer Advisor Stock Solution Recipe—quaternary phosphate buffer system.

adjustment to pH 6 → C and 10.5 → D). With this option, the user can construct self-made buffer compositions to enable the desired pH range. Buffer Advisor calculates the ionic strength (IS) as well as the buffering capacity (BC) for both buffer mixtures.

A: Water

B: n/a

C: pH = 6; IS = 22.5 mM; BC = 6.19 mM

D: pH = 10.5; IS = 0.717 mM; BC = 2.31 mM

## Method

**Table 1.** Quaternary phosphate-based buffer system—salt gradient chromatographic conditions.

Parameter	Value
Solvent	A: Water B: 1,700 mM sodium chloride C: 44.5 mM sodium phosphate monobasic D: 55 mM sodium phosphate dibasic
Gradient	Gradient from 0 to 30 minutes from 10 to 110 mM NaCl in 30 mM phosphate buffer, pH 6.8 with 500 mM NaCl washing step from 30 to 31 minutes Stop time: 31 minutes Post time: 15 minutes
Flow Rate	0.200 mL/min
Temperature	30 °C
Detection	280 nm 10 Hz
Injection	Injection volume: 4 µL Sample temperature: 8 °C Needle wash: 3 s in water

**Table 2.** Quaternary phosphate-based buffer system—pH gradient chromatographic conditions.

Parameter	Value
Solvent	A: Water B: 1,700 mM sodium chloride C: 44.5 mM sodium phosphate monobasic D: 55 mM sodium phosphate dibasic
Gradient	Gradient from 0 to 30 minutes from pH 7 to 8.4 in 30 mM phosphate buffer, pH 6.8 with 500 mM NaCl washing step from 30 to 31 minutes Stop time: 31 minutes Post time: 15 minutes
Flow Rate	0.200 mL/min
Temperature	30 °C
Detection	280 nm 10 Hz
Injection	Injection volume: 4 µL Sample temperature: 8 °C Needle wash: 3 s in water

**Note:** When using concentrated salt solutions as eluents, consider setting corresponding solvent types in the pump method. So, for example, for solvent B in the phosphate-buffered gradient with 1,700 mM NaCl, use *Sodium Chloride 1.5 M rather than Generic Aqueous or Water* in the solvent selection field in the pump method. High amounts of salts change the compressibility of the solvent, and hence using the preconfigured solvent tables enables best pump performance.

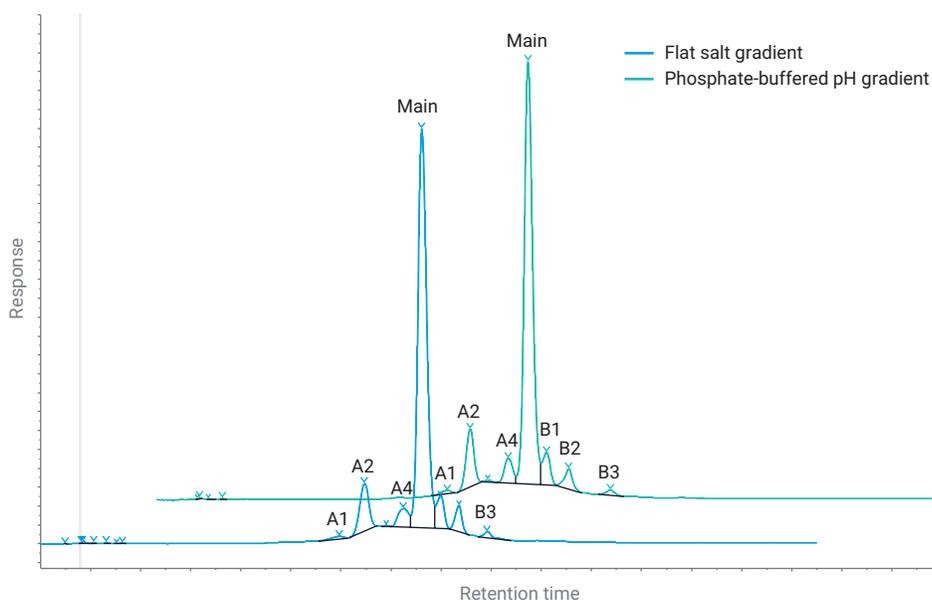
## Results and discussion

With Buffer Advisor, it is possible to calculate both salt as well as pH gradients. With the quaternary phosphate buffer described in the Experimental section of this application note, it is possible to calculate both versions for the separation of trastuzumab charge variants.

Figure 2 displays the overlay of two chromatograms, separating trastuzumab charge variants with a flat salt gradient (blue) and a phosphate-buffered pH gradient from pH 7 to 8.4 (green). The separations by salt and pH gradient are comparable, with slight improvements in resolution when using the pH gradient.

**Table 3.** Binary wide pH gradient buffer system/Farnan pH gradient.

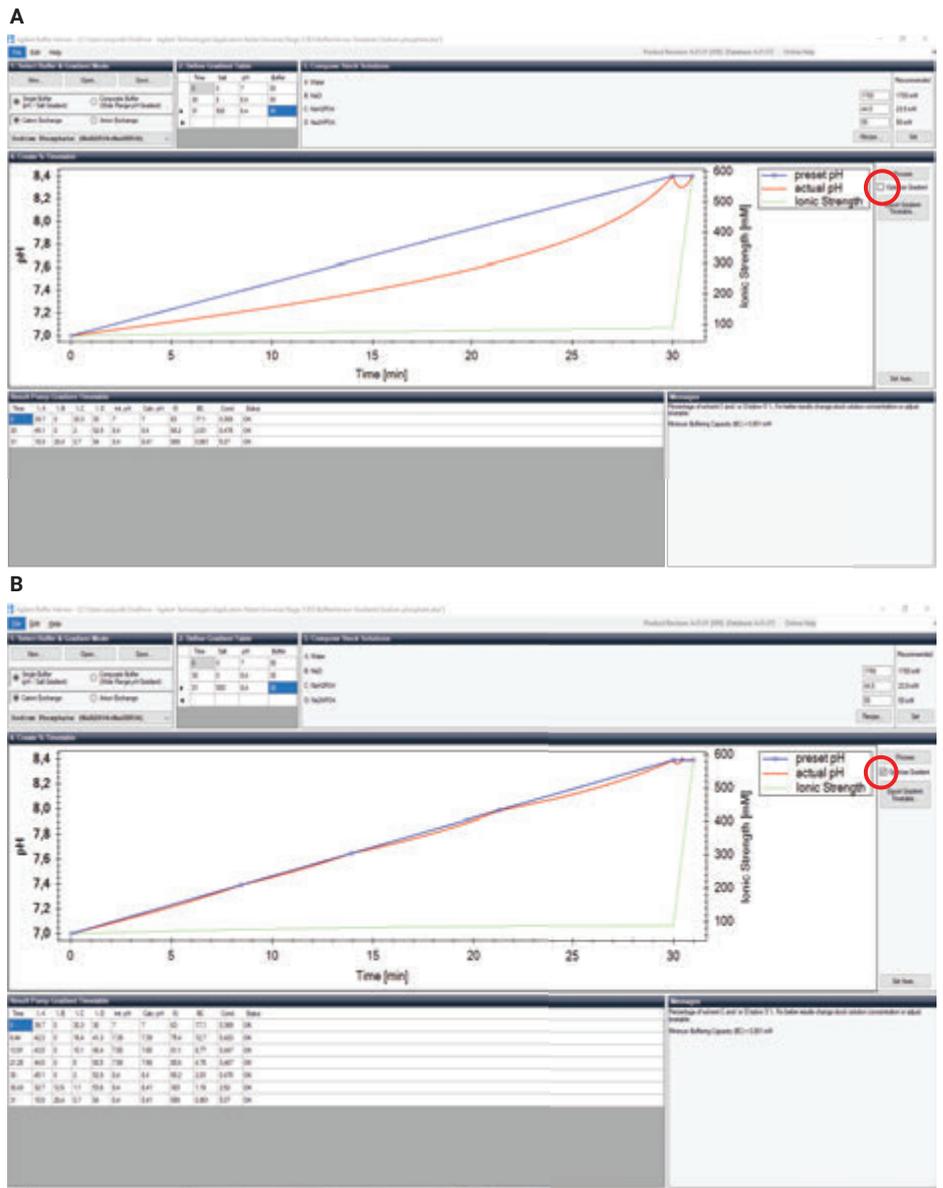
Parameter	Value
Solvent	A: n/a B: n/a C: pH = 6; IS = 22,4 mM; BC = 6,14 mM (Farnan Buffer <sup>1</sup> ) D: pH = 10,5; IS = 0,717 mM; BC = 2,31 mM (Farnan Buffer <sup>1</sup> )
Gradient	Trastuzumab gradient: Gradient from 0 to 50 minutes from pH 8.3 to 10 with a subsequent "wash" step from 51 to 55 minutes at pH 10.5 Stop time: 55 min Post time: 20 min NISTmAb gradient: Gradient from 0 to 45 minutes from pH 8.9 to 10.5 Stop time: 50 min Post time: 20 min
Flow Rate	0.200 mL/min
Temperature	30 °C
Detection	280 nm 10 Hz
Injection	Injection volume: 4 µL Sample temperature: 8 °C Needle wash: 3 s in water



**Figure 2.** Overlay of two chromatograms for the separation of charge variants with a flat salt gradient (blue) as well as a phosphate-buffered pH gradient from pH 7 to 8.4 (green).

One of the features of Buffer Advisor Software is the improved calculation of linear gradients (salt as well as pH) by adding additional gradient steps within the given gradient to enable perfect linearity without major deviations from the desired/preset pH. To enable this functionality, the **Optimize Gradient** box in the 4. Create % Timetable section in the Buffer Advisor user interface (UI) needs to be checked (see red circle in Figures 3A and 3B).

Figures 3A and B showcase the difference between the preset and the actual pH if the box is unchecked (A) and checked (B). With no further optimization from Buffer Advisor, the actual pH can deviate up to 0.4 units from the preset pH, which makes it difficult for the user to rely on the running gradient linearity. By checking the **Optimize Gradient** box (Figure 3B), additional steps are inserted into the original gradient to ensure linearity of the pH gradient. The **Result Pump Gradient Timetable** on the bottom left displays the additional inserted steps resulting in the actual gradient being as close as possible to the preset gradient. This gradient timetable can then be exported into the method in OpenLab for an easy transfer without additional time needed for typing.

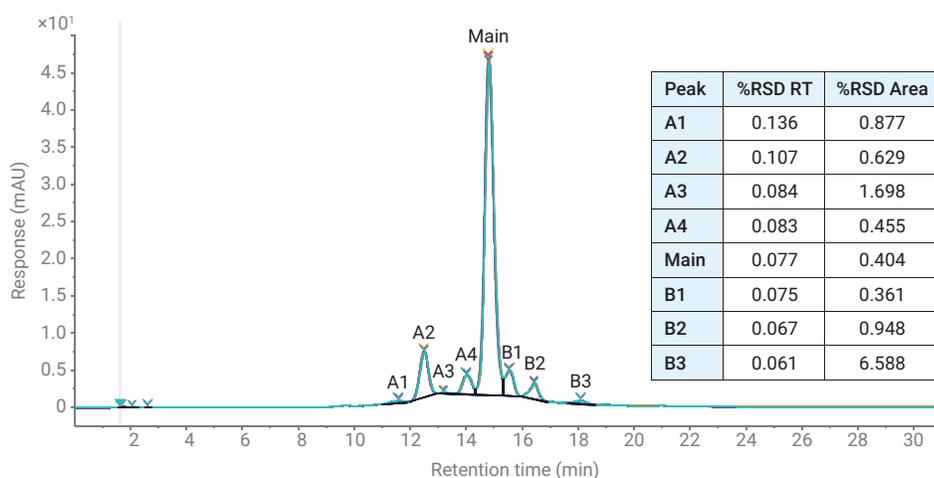


**Figure 3.** Optimize Gradient function in Agilent Buffer Advisor software to enable highly linear pH gradient. A shows no optimization, B displays the optimized gradient.

Figure 4 shows an overlay of seven subsequent runs of the charge variant analysis of trastuzumab with the phosphate-buffered pH gradient from pH 7 to 8.4. Excellent reproducibility was found for retention time (RT) and area with relative standard deviation (RSD) of less than 0.085% apart from the first two peaks. Due to the minimal area as well as height of the variants A3 and B3, the area reproducibility is higher than 1%.

Within the phosphate-buffered system, method development is limited, especially if the pI of the molecules of interest is not in the pH buffering range between 6 and 8. If the pI of the molecule is too high, elution is not possible using this buffer system. For example, the NIST mAb with a pI of 9.18<sup>6</sup> needs a different buffer system to enable elution from the CEX column.

A more generic approach is to use a wide-range pH gradient composed of more than one buffer system. This setup is also incorporated in Buffer Advisor Software under the name *Composite Buffer (Wide Range pH Gradient)*. The pH gradient with pH range 6.0 to 10.5 from Farnan and Moreno (2009)<sup>1</sup> is a suitable buffer system to analyse the charge variants of monoclonal antibodies. This system was further used and the method optimized for trastuzumab and NIST mAb.

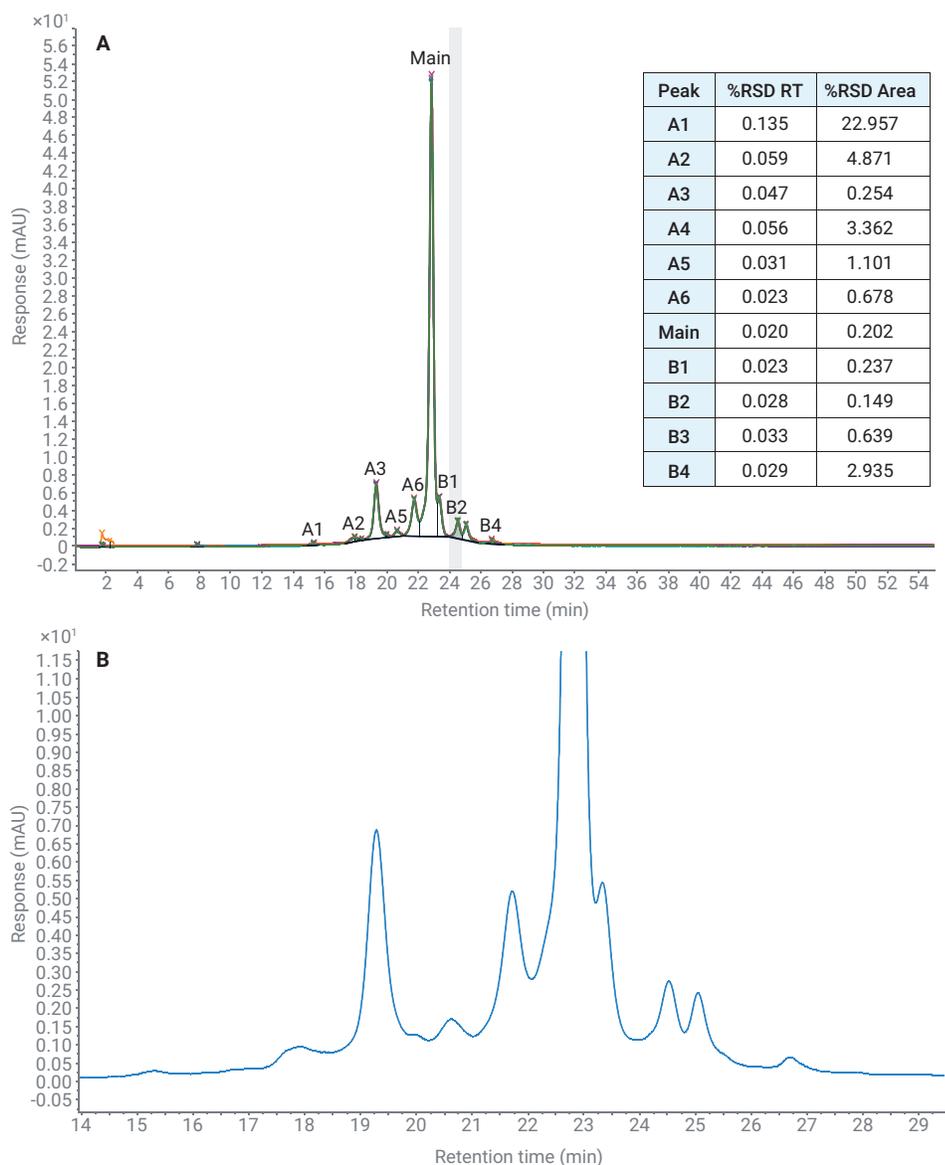


**Figure 4.** Overlay of seven consecutive runs of trastuzumab analyzed with phosphate-buffered pH gradient from pH 7 to 8.4 including the precision table for retention time (RT) and area.

Figure 5 shows the separation of trastuzumab charge variants using the wide-range pH gradient, narrowed from pH 8.3 to 10 to achieve optimal resolution. Compared to the phosphate-buffered pH gradient (see Figure 2), it was possible to resolve two more acidic variants—A1 to A6—eluting before, and one more basic variant eluting after the main peak. Especially the zoomed view in Figure 5B shows the excellent resolution of different charge variants around the main peak, with sharper peaks and enhanced resolving power compared to the shallow salt and pH gradient shown in Figure 2.

The precision of RT and area was evaluated for all resolved variants (see peak table in Figure 5). Even for extremely small peaks, the precision of RT was excellent, with values below 0.06% RSD except for the first variant A1. The area precision showed excellent values for most of the peaks except for the extremely small ones.

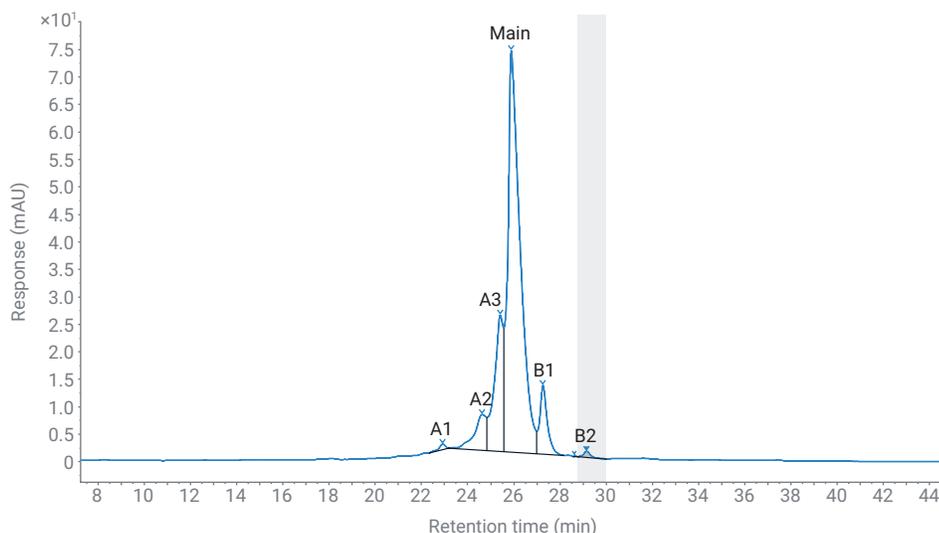
The pH gradient used by Farnan and Moreno<sup>1</sup> has also proven to be ideal for the analysis of the NISTmAb (see Figure 6). For the NISTmAb, the pH gradient was modified to a different pH range due to the different pI of the NISTmAb. With this developed shallow gradient from pH 8.9 to 10.5, it was possible to separate three acidic and two basic variants.



**Figure 5.** Overlay of seven consecutive runs of trastuzumab using a wide pH gradient from pH 8.3 to 10 including the precision table for retention time (RT) and area A. Zoomed view of a single injection B.

## Conclusion

The advantage of pH gradients over salt gradients was demonstrated for the analysis of monoclonal antibodies. pH gradients have been shown to outperform even shallow salt gradients with simplifying method development on the one hand and the generation of high-resolution chromatographic results on the other hand. While the potential of salt gradient method development is rather limited—changes in gradient slope just increase peak width with no further changes in resolution<sup>7</sup>—pH gradients reveal possibilities to further increase resolution and maintain sharp peaks. This was showcased by the analysis of trastuzumab and NIST mAb, especially by the use of the wide range pH gradient, based on Farnan and Moreno.<sup>1</sup> Buffer Advisor Software facilitated dynamic mixing of four stock solutions for the phosphate-buffered systems, preventing time-consuming buffer preparation hands-on time in the lab. In addition, the wide-range pH gradient could be easily calculated by Buffer Advisor. Hence, all methods—developed with Buffer Advisor Software and run on the Agilent 1260 Infinity II Prime Bio LC System with Flexible pump, with its completely iron-free sample flow path—delivered highly reliable and reproducible results.

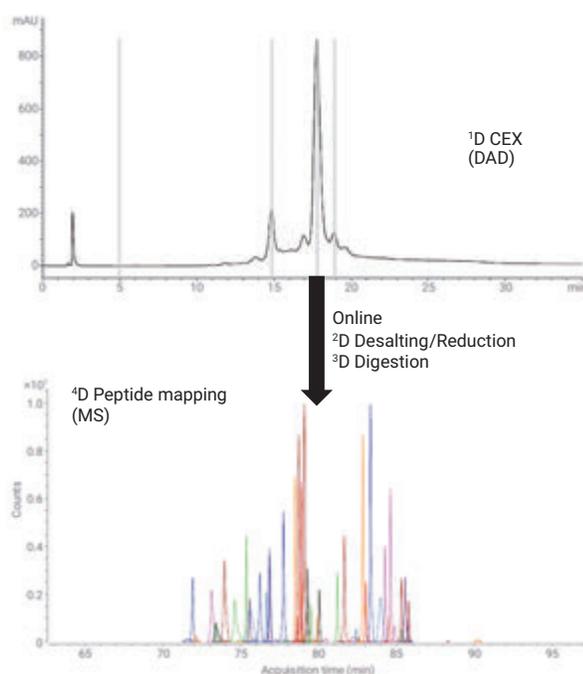


**Figure 6.** Separation of the NISTmAb with a pH gradient modified according to Farnan and Moreno<sup>1</sup> from pH 8.9 to 10.5.

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# Fully Automated Characterization of Monoclonal Antibody Charge Variants Using 4D-LC/MS



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## Abstract

This application note describes the fully automated and in-depth characterization of monoclonal antibody (mAb) charge variants by four-dimensional liquid chromatography/mass spectrometry (4D-LC/MS) using the Agilent InfinityLab 2D-LC Solution and the Agilent 6545 LC/Q-TOF system. Charge variants resolved by cation-exchange chromatography (CEX) are collected in loops installed on a multiple heart-cutting valve and consecutively subjected to online desalting, denaturation, reduction, and tryptic digestion prior to LC/MS-based peptide mapping.

## Introduction

Protein biopharmaceuticals have emerged as important therapeutics for the treatment of various diseases including cancer, cardiovascular diseases, diabetes, infection, inflammatory, and autoimmune disorders.<sup>1-3</sup> Protein biopharmaceuticals come in many flavors and include monoclonal antibodies (mAbs), antibody-drug conjugates (ADCs), fusion proteins, hormones, growth factors, cytokines, therapeutic enzymes, blood factors, vaccines, and anticoagulants. Given their obvious benefits in terms of safety and efficacy, these molecules have substantially reshaped the pharmaceutical market, and today, over 350 products have been approved for human use in the United States and the European Union.<sup>1-3</sup> This represents approximately one quarter of the total pharmaceutical market, with mAbs being the fastest growing class of pharmaceuticals.

Together with a huge therapeutic potential, these molecules come with an enormous, analytically demanding structural complexity.<sup>1,2</sup> In contrast to small molecule drugs, biopharmaceuticals are large (mAbs have an MW of approximately 150 kDa) and heterogeneous. They are the product of one or a couple of genes. However, hundreds of possible variants that differ in post-translational modifications (PTMs), amino acid sequence, higher-order structure, etc. may coexist, all making up the profile, safety, and efficacy of the product.<sup>1-3</sup> Consequently, their in-depth structural characterization involves a significant number of analytical tools, with chromatography (LC) and mass spectrometry (MS) at the forefront.

A key technology to study charge variants that might arise from PTMs such as asparagine deamidation, C-terminal lysine truncation, N-terminal cyclization (pyroglutamate formation), sialylation, etc. is CEX. In CEX, electrostatic interaction between the anionic groups of the stationary phase and cationic groups on the protein surface form the basis of the separation. The protein is loaded on the column at a mobile phase pH below its isoelectric point (pI), and elution is achieved using a salt or pH gradient. CEX buffers are typically composed of nonvolatile constituents, making these methods incompatible with MS. Peak identification is a laborious task involving peak collection and desalting prior to MS analysis.<sup>4</sup> With the recent introduction of commercial and robust 2D-LC instrumentation, this series of events is now commonly performed in an online automated manner.<sup>5-9</sup> Peaks eluting from the CEX column are stored in loops and subjected to online desalting using reversed-phase (RPLC) or size exclusion chromatography (SEC) prior to MS measurement. Both comprehensive (LC×LC) and (multiple) heart-cutting 2D-LC (LC-LC) have been used.<sup>5-9</sup> To unambiguously identify CEX peaks, however, peptide mapping is required. While protein measurement is indicative of identity and highlights dominant modifications with mass differences beyond the mass accuracy of the MS instrument, it typically does not provide the actual amino acid sequence, nor does it allow us to localize modifications. Addressing the latter, and inspired by previous work,<sup>10-12</sup> the current application note describes a fully automated online 4D-LC/MS setup incorporating first dimension (1D) CEX, peak collection, 2D desalting, denaturation, reduction, 3D trypsin digestion, and 4D RPLC/MS-based peptide mapping for the in-depth characterization of mAb charge variants.

## Experimental

### Materials

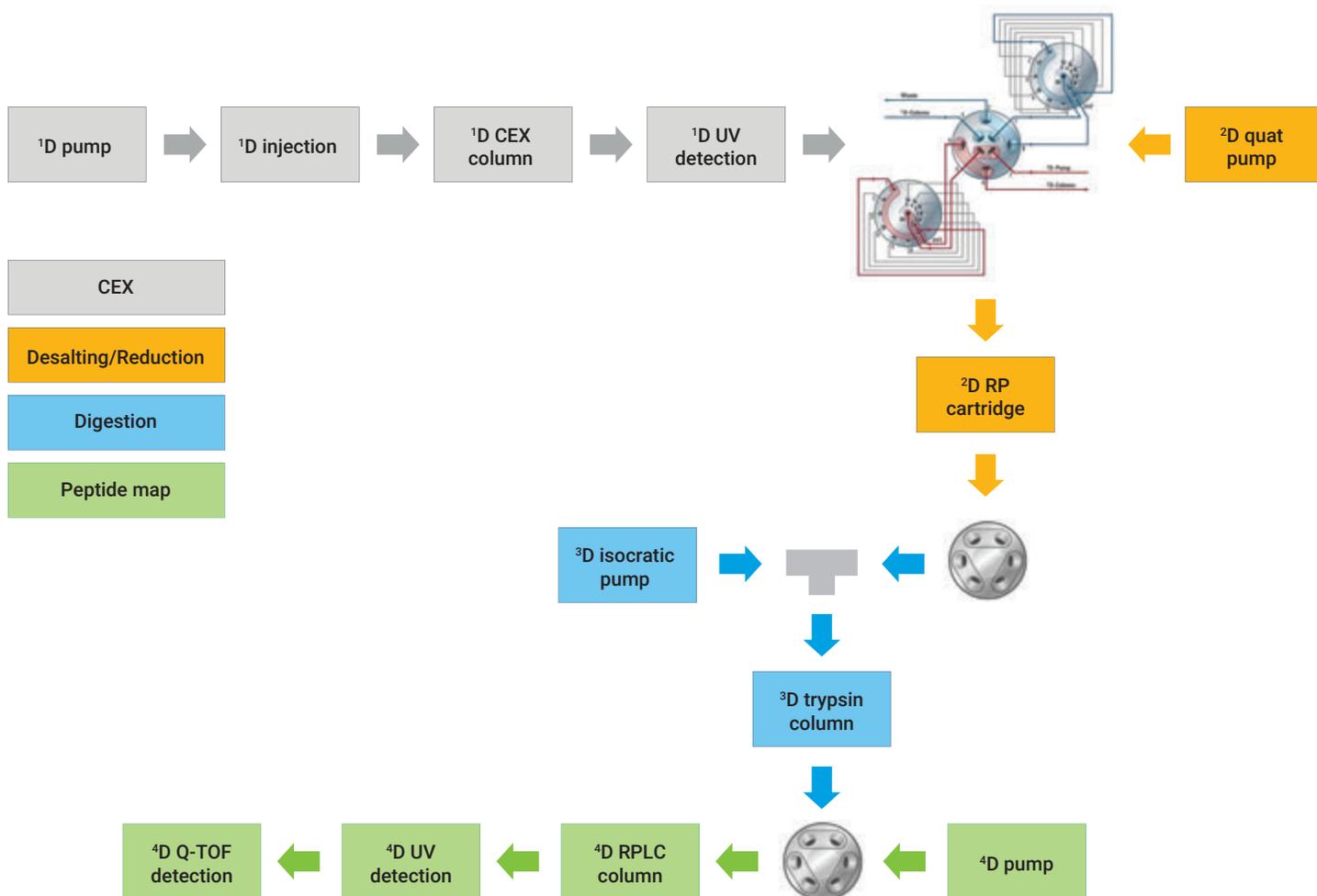
Acetonitrile (HPLC-S), water (ULC/MS), and formic acid (ULC/MS) were obtained from Biosolve (Valkenswaard, The Netherlands). NaH<sub>2</sub>PO<sub>4</sub>, Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, NaCl, NH<sub>4</sub>HCO<sub>3</sub>, Tris base, and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO, USA). UltraPure Tris-HCl pH 7.5 was purchased from ThermoFisher Scientific (Waltham, MA, USA). Type I water was produced from tap water by an arium pro Ultrapure Lab Water System from Sartorius (Göttingen, Germany). Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland).

### Sample preparation

Trastuzumab was diluted to 7 mg/mL in 1D CEX mobile phase A (MPA: 10 mM sodium phosphate pH 7.65). Deamidation was induced by incubating trastuzumab at 37 °C for 3 days in high pH conditions (100 mM Tris pH 9.0) and subsequently buffer-exchanged to 7 mg/mL in 1D CEX mobile phase A.

### Instrumentation

An Agilent 1290 Infinity II 2D-LC system equipped with the multiple heart-cutting option, an additional Agilent 1260 Infinity II quaternary pump and Agilent 1260 Infinity II isocratic pump, two additional 2-position/6-port valves, and a zero dead volume T-piece were used. Stainless steel tubing with an internal diameter of 0.12 mm was applied. The configuration is schematically represented in Figure 1 and summarized in this application note. Diode array detection (DAD) was used in the first (CEX) and fourth dimension (RPLC). Additionally, an Agilent 6545 LC/Q-TOF with a Jet Stream ESI source was used for detection after the fourth and final dimension.



**Figure 1.** 4D-LC/MS configuration incorporating 1D CEX separation and charge variant peak collection using multiple heart-cutting, 2D RPLC-based desalting, denaturation, reduction, 3D trypsin digestion, and 4D RPLC-MS based peptide mapping.

## Configuration

### 1D: Cation-exchange chromatography

- G7120A Agilent 1290 Infinity II high-speed pump
- G7167B Agilent 1290 Infinity II multisampler with sample thermostat (option 101)
- G7116B Agilent 1290 Infinity II multicolumn thermostat (MCT) with valve drive installed (option 058) equipped with an Agilent InfinityLab quick change 2-position/6-port valve, 1300 bar (G4231C)

- G7117B Agilent 1290 Infinity II diode array detector with a 3.7 mm HDR InfinityLab Max-Light cartridge cell (G4212-60032)

**Note:** A short 3.7 mm detector flow cell was installed to reduce the signal intensity and prevent saturation of the UV signal.

### 2D-LC with multiple heart-cutting

- Agilent 1290 Infinity valve drive (G1170A) with 2D-LC valve (G4236A)
- Two Agilent 1290 Infinity valve drives (G1170A) with multiple heart-cutting valves (G4242-64000) equipped with 40 µL loops

**2D:** Reversed-phase chromatography for desalting, denaturation, and reduction  
G7111B Agilent 1260 Infinity II quaternary pump with active inlet valve (AIV) (option 032)

**3D:** Trypsin digestion

- 0100-0969 ZDV T-piece
- G7110B Agilent 1260 Infinity II isocratic pump

**<sup>4</sup>D:** Reversed-phase chromatography for peptide mapping

- G7120A Agilent 1290 Infinity II high-speed pump
- G7116B Agilent 1290 Infinity II MCT with valve drive installed (option 058) equipped with an Agilent InfinityLab quick change 2-position/6-port valve, 1300 bar (G4231C)
- G7117B Agilent 1290 Infinity II DAD with a 10 mm InfinityLab Max-Light cartridge cell (G4212-60008)
- G6545A Agilent 6545 LC/Q-TOF with Jet Stream ESI source

**Note:** Orachrom StyrosZyme TPCK-Trypsin and Agilent AdvanceBio peptide mapping columns are both contained in different zones of one single column compartment and maintained at 40 and 60 °C, respectively.

#### Software

- Agilent OpenLab CDS ChemStation revision C.01.07 SR4 [505]
- 2D-LC add-on software revision A.01.04 [017]
- Agilent MassHunter for instrument control (B.09.00)
- Agilent MassHunter with BioConfirm add-on for data analysis (B.07.00)

#### Method

<sup>1</sup>D and <sup>4</sup>D were configured in the 2D-LC software, while <sup>2</sup>D and <sup>3</sup>D were controlled in a regular method setup and were programmed as repetitive events. The cycle time of these events was 110 minutes, identical to the <sup>4</sup>D cycle time programmed in the 2D-LC software. MassHunter acquisition was triggered by a remote start from the 2D-LC system.

Four heart-cuts were taken across the CEX analysis. The first heart-cut at 4.8 minutes is a blank cut, which enables preconditioning of all dimensions before the analysis of the actual CEX heart-cuts of interest.

<b><sup>1</sup>D: Cation-Exchange Chromatography</b>			
Column	Agilent Bio MAb, nonporous (2.1 mm × 250 mm, 5 µm) (p/n 5190-2411)		
Temperature	30 °C		
Mobile phase A	10 mM sodium phosphate, pH 7.65		
Mobile phase B	10 mM sodium phosphate, pH 7.65 + 100 mM NaCl		
Flow rate	0.2 mL/min		
Gradient	Time (min)	B (%)	CEX analysis
	0	5	
	36	70	
	36.5	100	
	46	100	
	46.5	5	
60	5		
Injection	100 µg		
Detection	220 and 280 nm		
Peak Width	> 0.025 min (10 Hz)		

<b>Multiple Heart-Cutting <sup>1</sup>D &gt; <sup>2</sup>D</b>	
<b>Sampling Timetable Trastuzumab</b>	
Cut	Time (min)
1 – Blank	4.80
2 – Pre-peak	14.93
3 – Main Peak	17.55
4 – Post-peak	18.99

<b><sup>2</sup>D: Reversed-Phase Chromatography for Desalting, Denaturation, and Reduction (Manually Entered Repetitive Event)</b>						
Column	Polymer-based desalting cartridge, 2.1 × 10 mm					
Temperature	23 °C					
Mobile Phase A	0.1% (v/v) formic acid in water					
Mobile Phase B	0.1% (v/v) formic acid in acetonitrile					
Mobile Phase C	20 mM DTT in 100 mM Tris-HCl, pH 7.5					
Gradient	Time (min)	A (%)	B (%)	C (%)	Flow (mL/min)	
	10	99	1	0	0.5	Desalting and focusing
	10.01	0	0	100	0.2	Reduction
	20	0	0	100	0.2	
	20.01	99	1	0	0.5	Desalting and elution
	25	99	1	0	---	
	25.01	40	60	0	---	
	27	40	60	0	0.5	
	27.01	40	60	0	0.015	
	68	40	60	0	0.015	Elution and digestion
	68.01	0	100	0	0.5	
	85	0	100	0	---	
	95	99	1	0	---	
120	99	1	0	---		
Valve	27 min: Pos 1 → Pos 2 (start trypsin digestion) 67 min: Pos 2 → Pos 1 (start peptide mapping)					

<sup>3</sup> D: Trypsin Digestion (Manually Entered Repetitive Event)			
Column	Orachrom StyrosZyme TPCK-Trypsin PEEK (2.1 × 150 mm)		
Temperature	40 °C		
Mobile Phase	50 mM NH <sub>4</sub> HCO <sub>3</sub> , pH 8		
Gradient	Time (min)	Flow (mL/min)	
	25	0.06	
	25.01	0.135	Digestion
	67	0.135	
	67.01	0.06	
135	0.06		
Valve	27 min: Pos 1 -> Pos 2 (start trypsin digestion) 67 min: Pos 2 -> Pos 1 (start peptide mapping)		

## Data processing

Measured signals were matched onto the trastuzumab light- and heavy-chain sequences using the BioConfirm algorithm incorporated in the MassHunter software. Mass tolerance for matching experimental data onto the sequence was set at 8 ppm. Extracted ion chromatograms (EICs) obtained at 20 ppm mass accuracy were used to monitor PTMs such as deamidation.

## Results and discussion

A scheme of the fully automated online 4D-LC/MS protein analyzer, incorporating CEX, peak collection, desalting, denaturation, reduction, trypsin digestion, and peptide mapping, is shown in Figure 1. CEX peaks are collected in 40 µL loops installed on a multiple heart-cutting valve and transferred one by one to a polymeric RP cartridge where desalting, denaturation, and reduction take place. The reduced mAb, trapped on the cartridge, is subsequently eluted into the trypsin column by raising the acetonitrile concentration. Using a T-piece, trypsin digestion buffer is mixed with the reversed-phase mobile phase to have optimal digestion conditions and to reduce the acetonitrile concentration. During the digestion, the trypsin column

<sup>4</sup> D: Reversed-Phase Chromatography for Peptide Mapping (Repetitive Event Controlled by 2D-LC Software)			
Column	Agilent AdvanceBio peptide mapping (2.1 × 150 mm × 2.7 µm) (p/n 651750-902)		
Temperature	60 °C		
Mobile Phase A	0.1% (v/v) formic acid in water		
Mobile Phase B	0.1% (v/v) formic acid in acetonitrile		
Flow Rate	0.4 mL/min		
Gradient	Time (min)	B (%)	
	0	1	
	8.5	1	
	9	100	
	15	100	
	16	1	
	20	1	Load digest on peptide mapping column
	64	1	
	64	1	Peptide mapping
	97	45	
	98	100	
	103	100	
	104	1	
110	1		
DAD Detection	214 and 280 nm		
Peak Width	> 0.025 min (10 Hz)		
MS Detection			
Source			
Positive Ionization			
Drying Gas Temperature	300 °C		
Drying Gas Flow	8 L/min		
Nebulizer Pressure	35 psi		
Sheath Gas Temperature	350 °C		
Sheath Gas Flow	8 L/min		
Capillary Voltage	3,500 V		
Nozzle Voltage	1,000 V		
Fragmentor	175 V		
Acquisition			
Mode	Extended dynamic range (2 GHz)		
Data Acquisition Range	m/z 100 to 3,200		
	1 spectrum/s		
	Centroid acquisition		
Switch diverter valve to MS after 67 minutes			

is in-line with the peptide mapping column, and generated peptides are focused at the head of the RPLC column. After 20 minutes, a valve switch initiates the elution of the digest into the MS.

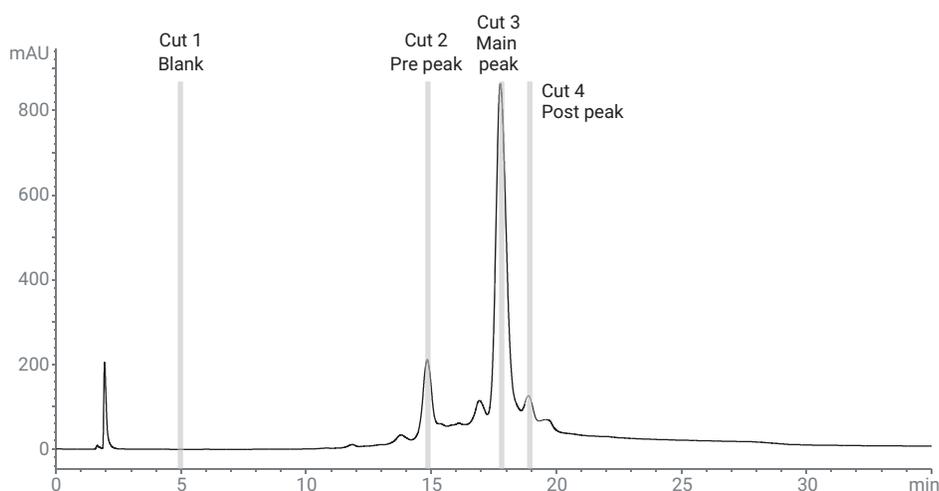
The CEX chromatogram of the mAb trastuzumab is shown in Figure 2. Trastuzumab, commercialized as Herceptin, is a humanized IgG1 binding the HER2 receptor, thereby finding use in the treatment of HER2 positive metastatic breast cancer. With a pI of 8.45, the mAb is positively charged at the CEX mobile phase pH, thereby governing interaction with the negatively charged chromatographic resin. Upon eluting the mAb using a NaCl salt gradient, various charge variants were revealed, which were subsequently subjected to online peptide mapping. Figure 3A schematically presents the 4D-LC/MS experiment involving the analysis of three CEX peaks (pre-, main-, and post-peaks) and a CEX blank as shown in Figure 2. Figure 3B zooms in on the pressure and DAD profiles of one cycle and shows the desalting, denaturation, reduction, digestion, and peptide mapping of the main CEX peak. Over 90% sequence coverage could be obtained. Peptides identified are shown in Table 1, and an overlay of the LC/MS compound chromatograms is provided in Figure 4. Peptides not covered are typically small and/or hydrophilic and are not focused at the head of the peptide mapping column during the digestion. For this reason, they are diverted to waste.

Next to sequence information, peptide

mapping also reveals modifications and modification sites. Figure 5 shows the online peptide mapping of the CEX pre-, main-, and post-peak, and in particular the EICs of two peptides (i.e., light-chain peptide ASQDVNTAVAWYQQKPGK (LC 25–42) containing a potential deamidation site at position 30, and heavy-chain peptide WGGDGFYAMDYWGQGLTVSSASTK (HC 99–124) containing a potential isomerization site at position 102). From the data, it could be deduced that the pre-peak corresponds to a deamidated variant, with asparagine converted to aspartate at position 30 on one of the light chains. It could also be demonstrated that the post-peak carries an isoaspartate at position 102 on one of the heavy chains. This is clearly visualized by the peak doublets corresponding to the modified and nonmodified variants. These results are in accordance with those reported by Harris *et al.*, who performed offline

fraction collection and peptide mapping on Herceptin acidic and basic variants.<sup>4</sup>

The same experiment was performed on a high-pH stressed Herceptin sample (Figure 6). Such conditions are known to induce deamidation, thereby rendering the mAb more acidic. The CEX profile presented in Figure 6 shows an acidic shift, and the peptide map data of CEX peaks 1 and 2 show a double deamidated variant, with both light chains deamidated at position 30. CEX peaks 3 and 4 correspond to a single deamidation event, with one light chain deamidated at position 30. The difference between peaks 1 and 2, and 3 and 4 originate from another deamidation, this time at position 387 in the heavy chain. This deamidation site appears in two peptides (one fully cleaved and one miscleaved) that are apparently digested differently when a deamidation exists.



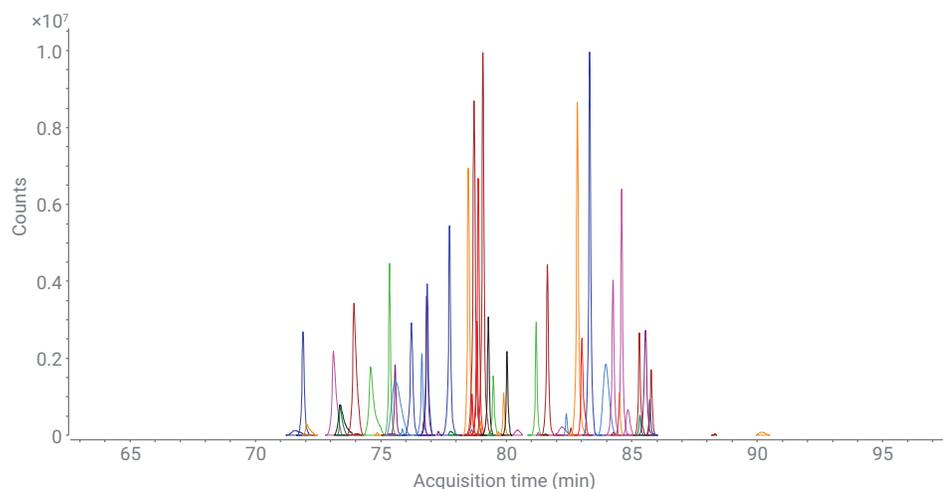
**Figure 2.** CEX chromatogram of the monoclonal antibody trastuzumab. Conditions according to reference 13. Heart-cuts taken are indicated in gray.



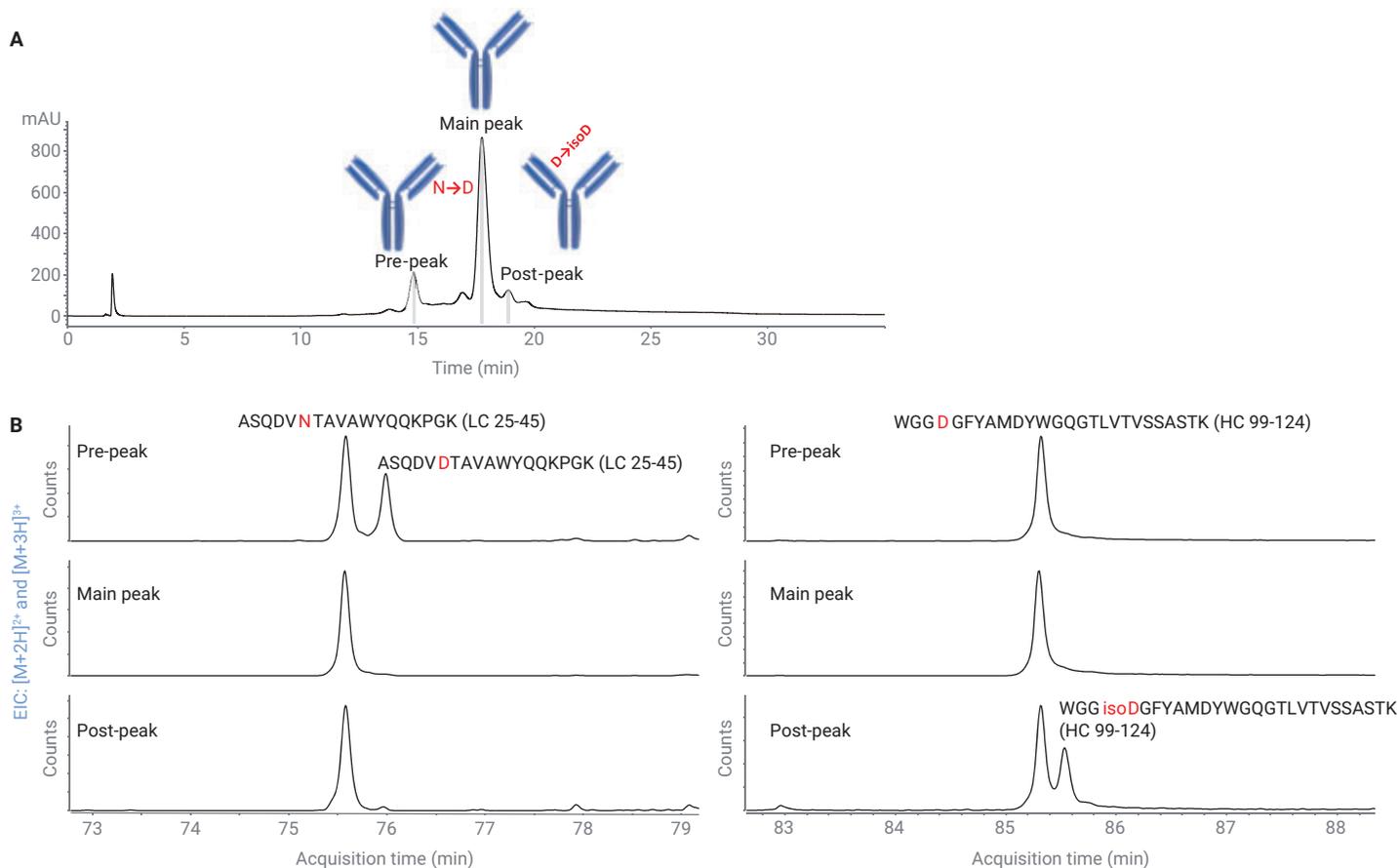
**Table 1.** Peptides identified in the CEX main peak following online RPLC/MS-based peptide mapping.

RT	Mass	Vol	Vol %	Sequence	Seq Loc.	Tgt. Seq. Mass	Diff. (ppm)	Missed Cleavage
79.1	1880.9972	66635668	6.61	EVQLVESGGGLVQPGGSLR	HC(001-019)	1880.9956	0.8	0
76.8	1109.5539	25727274	2.55	LSCAASGFNIK	HC(020-030)	1109.5539	0.0	0
81.2	2180.0864	16055279	1.59	LSCAASGFNIKDTYIHVWR	HC(020-038)	2180.0837	1.2	1
76.6	1088.5410	13618352	1.35	DTYIHVWR	HC(031-038)	1088.5403	0.6	0
76.2	829.4442	24354704	2.42	GLEWVAR	HC(044-050)	829.4446	-0.5	0
71.9	1083.5360	19879112	1.97	IYPTNGYTR	HC(051-059)	1083.5349	1.0	0
72.1	1181.6059	3708836	0.37	GRFTISADTSK	HC(066-076)	1181.6041	1.6	1
73.1	968.4819	26423682	2.62	FTISADTSK	HC(068-076)	968.4815	0.4	0
79.3	2260.1184	317550	0.03	FTISADTSKNTAYLQMNSLR	HC(068-087)	2260.1158	1.1	1
81.3	3518.6474	320893	0.03	FTISADTSKNTAYLQMNSLRAEDTAVYYCSR	HC(068-098)	3518.6446	0.8	2
76.8	1309.6451	24858112	2.47	NTAYLQMNSLR	HC(077-087)	1309.6449	0.1	0
79.9	2568.1769	5903991	0.59	NTAYLQMNSLRAEDTAVYYCSR	HC(077-098)	2568.1737	1.2	1
71.6	1276.5392	2994056	0.30	AEDTAVYYCSR	HC(088-098)	1276.5394	-0.1	0
85.3	2783.2545	16863744	1.67	WGGDGFYAMDYWGQGLTVTVSSASTK	HC(099-124)	2783.2537	0.3	0
78.7	1185.6398	68405792	6.79	GPSVFPLAPSSK	HC(125-136)	1185.6394	0.4	0
77.7	1263.6494	36588096	3.63	STSGGTAALGCLVK	HC(137-150)	1263.6493	0.1	0
88.3	6655.2898	221285	0.02	DYFPEPVTVSWNSGALTSGVHTFPAVLQSS GLYSLSSVTVPSSSLGTQTYICNVNHKPSNTK	HC(151-213)	6655.2857	0.6	0
79.8	1374.7171	214690	0.02	VDKKEVPEKSCDK	HC(214-225)	1374.7177	-0.4	3
84.9	2729.4093	7103687	0.71	THTCPPCPAPELLGSPVFLFPPKPK	HC(226-251)	2729.4073	0.7	0
73.4	834.4277	13166738	1.31	DTLMISR	HC(252-258)	834.4269	1.0	0
82.0	2897.4175	220680	0.02	DTLMISRTEPVTCTVVVDVSHEDPEVK	HC(252-277)	2897.4151	0.9	1
84.3	4556.2041	547132	0.05	DTLMISRTEPVTCTVVVDVSHEDPEVKFNWYVDGVEVHNAK	HC(252-291)	4556.1992	1.1	2
79.3	2081.0013	17858876	1.77	TPEVTCVVVDVSHEDPEVK	HC(259-277)	2080.9987	1.2	0
83.0	3739.7881	18901388	1.88	TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	HC(259-291)	3739.7828	1.4	1
78.6	1676.7966	5991668	0.59	FNWYVDGVEVHNAK	HC(278-291)	1676.7947	1.1	0
84.6	1807.0008	38113724	3.78	VVSVLTVLHQDWLNGK	HC(305-320)	1806.9992	0.9	0
83.3	2227.2022	63210428	6.27	VVSVLTVLHQDWLNGKEYK	HC(305-323)	2227.2001	0.9	1
82.4	2458.3080	2959244	0.29	VVSVLTVLHQDWLNGKEYKCK	HC(305-325)	2458.3043	1.5	2
81.6	2886.5495	183447	0.02	VVSVLTVLHQDWLNGKEYKCKVSNK	HC(305-329)	2886.5426	2.4	3
73.9	837.4964	38694668	3.84	ALPAPIEK	HC(330-337)	837.4960	0.5	0
75.4	1285.6662	875645	0.09	EPQVYTLPPSR	HC(348-358)	1285.6667	-0.4	0
75.6	1903.9366	36378636	3.61	EPQVYTLPPSREEMTK	HC(348-363)	1903.9350	0.8	1
80.5	2989.5263	1849832	0.18	EPQVYTLPPSREEMTKNQVSLTCLVK	HC(348-373)	2989.5253	0.3	2
78.4	1721.8701	179925	0.02	EEMTKNQVSLTCLVK	HC(359-373)	1721.8692	0.6	1
78.9	1103.6013	45037560	4.47	NQVSLTCLVK	HC(364-373)	1103.6009	0.4	0
81.6	2543.1245	29221288	2.90	GFYPSDIAVEWESNGQPENNYK	HC(374-395)	2543.1241	0.2	0
85.7	4398.0307	5032842	0.50	GFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSK	HC(374-412)	4398.0281	0.6	1
85.9	4954.3531	225838	0.02	GFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDK	HC(374-417)	4954.3502	0.6	2
82.8	1872.9144	57538216	5.71	TTPPVLDSDGSFFLYSK	HC(396-412)	1872.9146	-0.1	0
82.6	2429.2370	970108	0.10	TTPPVLDSDGSFFLYSKLTVDK	HC(396-417)	2429.2366	0.2	1
77.8	2986.3744	1042270	0.10	SRWQQGNVFSCSVMHEALHNHYTQK	HC(418-442)	2986.3715	1.0	1
78.5	2743.2427	46302832	4.60	WQQGNVFSCSVMHEALHNHYTQK	HC(420-442)	2743.2384	1.6	0
74.6	659.3487	26985572	2.68	SLSLSPG	HC(443-449)	659.3490	-0.5	0
76.7	1877.8787	2655889	0.26	DIQMTQSPSSLSASVGDR	LC(001-018)	1877.8789	-0.1	0
79.5	2551.2398	9011163	0.89	DIQMTQSPSSLSASVGRVITICR	LC(001-024)	2551.2371	1.1	1

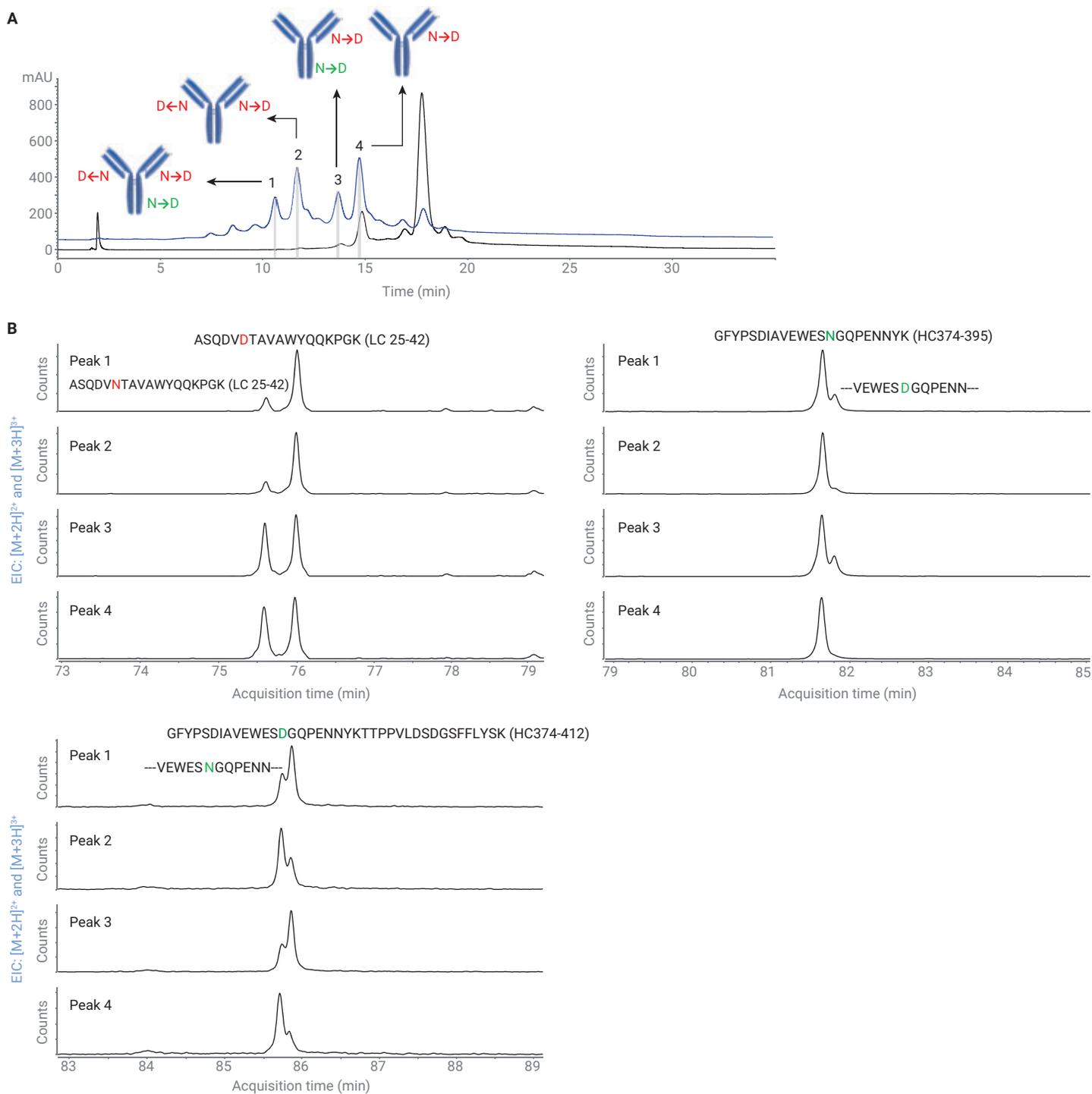
RT	Mass	Vol	Vol %	Sequence	Seq Loc.	Tgt. Seq. Mass	Diff. (ppm)	Missed Cleavage
75.6	1989.9932	10501849	1.04	ASQDVNTAVAWYQKPGK	LC(025-042)	1989.9908	1.2	0
74.8	2286.1771	370620	0.04	ASQDVNTAVAWYQKPGKAPK	LC(025-045)	2286.1757	0.6	1
84.3	1771.9519	27274394	2.71	LLIYSASFLYSGVPSR	LC(046-061)	1771.9509	0.6	0
85.8	4129.8936	10160642	1.01	SGTDFTLTISLQPEDFATYYCQHYTTPPTFGQGK	LC(067-103)	4129.8892	1.1	0
85.3	4599.1803	2980494	0.30	SGTDFTLTISLQPEDFATYYCQHYTTPPTFGQGTKEIK	LC(067-107)	4599.1792	0.2	1
84.5	4755.2888	6157193	0.61	SGTDFTLTISLQPEDFATYYCQHYTTPPTFGQGTKEIKR	LC(067-108)	4755.2803	1.8	2
82.2	2101.1217	3894068	0.39	RTVAAPSVFIFPPSDEQLK	LC(108-126)	2101.1208	0.4	1
84.0	1945.0220	32666390	3.24	TVAAPSVFIFPPSDEQLK	LC(109-126)	1945.0197	1.2	0
90.2	3666.8789	1457746	0.14	TVAAPSVFIFPPSDEQLKSGTASVCLLNFFYPR	LC(109-142)	3666.8756	0.9	1
85.5	1739.8676	23403588	2.32	SGTASVCLLNFFYPR	LC(127-142)	1739.8665	0.6	0
75.9	2676.2628	852142	0.08	VQWKVDNALQSGNSQESVTEQDSK	LC(146-169)	2676.2627	0.0	1
80.0	4160.0087	11663837	1.16	VQWKVDNALQSGNSQESVTEQDSKDYSLSTLTLTK	LC(146-183)	4160.0033	1.3	2
79.7	4766.2746	403170	0.04	VQWKVDNALQSGNSQESVTEQDSKDYSLSTLTLTKADYEK	LC(146-188)	4766.2683	1.3	3
78.8	3618.7073	16391463	1.63	VDNALQSGNSQESVTEQDSKDYSLSTLTLTK	LC(150-183)	3618.7021	1.5	1
78.6	4224.9705	725845	0.07	VDNALQSGNSQESVTEQDSKDYSLSTLTLTKADYEK	LC(150-188)	4224.9670	0.8	2
77.3	4490.1265	478190	0.05	VDNALQSGNSQESVTEQDSKDYSLSTLTLTKADYEKHK	LC(150-190)	4490.1209	1.3	3
78.9	6290.0188	328711	0.03	VDNALQSGNSQESVTEQDSKDYSLSTLTLTKADYEKHKVYACEVTHQGLSSPVTK	LC(150-207)	6290.0085	1.6	4
79.0	1501.7515	2071466	0.21	DYSLSTLTLTK	LC(170-183)	1501.7512	0.2	0
74.0	2689.3218	547667	0.05	ADYEKHKVYACEVTHQGLSSPVTK	LC(184-207)	2689.3170	1.8	2
73.4	2083.0562	6116953	0.61	HKVYACEVTHQGLSSPVTK	LC(189-207)	2083.0521	2.0	1
75.3	1817.8988	25691042	2.55	VYACEVTHQGLSSPVTK	LC(191-207)	1817.8982	0.3	0



**Figure 4.** Overlaid RPLC/MS compound chromatograms of MS-identified peptides in the CEX main peak following online peptide mapping.



**Figure 5.** Online peptide mapping of trastuzumab CEX pre-, main-, and post-peaks. (A) the CEX chromatogram and (B) the extracted ion chromatograms of light-chain peptide ASQDV $N$ TAVAWYQQKPGK (LC 25–42), deamidated light-chain peptide ASQDV $D$ TAVAWYQQKPGK (LC 25–42), heavy-chain peptide WGG $D$ GFYAMDYWGQGLTVSSASTK (HC 99–124) and isomerized heavy-chain peptide WGG $isoD$ GFYAMDYWGQGLTVSSASTK (HC 99–124).



**Figure 6.** Online peptide mapping of high-pH stressed trastuzumab CEX peaks 1, 2, 3, and 4. (A) the overlaid CEX chromatograms of nonstressed and high-pH stressed trastuzumab and (B) the extracted ion chromatograms of light-chain peptide ASQD**V**NTAWAWYQQKPGK (LC 25–42), deamidated light-chain peptide ASQD**V**NTAWAWYQQKPGK (LC 25–42), heavy-chain peptide GFYPSDIAVEWES**N**GQPENNYK (HC 374–395), deamidated heavy-chain peptide GFYPSDIAVEWES**D**GQPENNYK (HC 374–395), heavy-chain peptide GFYPSDIAVEWES**N**GQPENNYKTPPVLDSDGSFFLYSK (HC374–412), and deamidated heavy-chain peptide GFYPSDIAVEWES**D**GQPENNYKTPPVLDSDGSFFLYSK (HC 374–412).

## Conclusion

A fully automated 4D-LC/MS protein analyzer incorporating <sup>1</sup>D CEX separation and charge-variant peak collection using multiple heart-cutting, <sup>2</sup>D RPLC-based desalting, denaturation, reduction, <sup>3</sup>D trypsin digestion, and <sup>4</sup>D RPLC/MS-based peptide mapping was described and successfully applied to characterize acidic and basic variants observed in the CEX profile of nonstressed and high-pH stressed trastuzumab. This multidimensional system is based on the InfinityLab 2D-LC Solution and the 6545 LC/Q-TOF system. A variant of this 4D-LC/MS design can readily be configured by replacing CEX in the first dimension by Protein A affinity chromatography, size exclusion chromatography, hydrophobic interaction chromatography, etc.

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## Two-In-One Bioprocess Analytics

Combining mAb titer determination and spent media analysis by internal valve switching using the Agilent 1260 Infinity II Bio-Inert LC and InfinityLab LC/MSD iQ



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### Abstract

The analysis of critical process parameters (CPP) and critical quality attributes (CQA) plays an important role in monitoring and ensuring optimal process yields and product quality during the production of biopharmaceuticals. HPLC methods are often used for the analysis of these parameters. However, cost, lab space, and workload are crucial for the implementation of such tools. This application note demonstrates the use of an Agilent 1260 Infinity II Bio-Inert LC System coupled to an Agilent InfinityLab LC/MSD iQ with an automated column selection valve for spent media analysis and protein A-based titer determination. By applying hydrophilic interaction liquid chromatography (HILIC) with smart MS detection, excellent linearity, sensitivity, and selectivity for important cell culture medium components were achieved. The quaternary pump used in the 1260 Infinity II Bio-Inert LC System enabled the subsequential analysis of the monoclonal antibody (mAb) product with UV detection, without manual reconfiguration of the instrument. All necessary maintenance operations such as column switching, washing, or equilibration could be performed using Agilent OpenLab CDS 2 software, which can be fully qualified to meet the requirements of the FDA regarding 21 CFR part 11 compliance.

## Introduction

The production of biopharmaceuticals such as mAbs is mainly performed by bioreactor cultivations of mammalian cell lines such as Chinese hamster ovary (CHO) cells. These production hosts require numerous nutrients such as sugars, amino acids, vitamins, and growth factors for optimal growth and yields.<sup>1</sup> The analysis of the process and product is encouraged by the International Council for Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) through concepts such as quality by design (QbD), which aims to build quality into the process instead of testing the quality of the final product.<sup>2</sup> Various analytical methods can be used for upstream/downstream processes or quality control as online, at-line, or offline tools. Important parameters for monitoring are nutrient consumption, product formation, and product quality (CQA).<sup>1</sup> Since lab space is limited, and the preparation or switching of analytical methods can be time-consuming, there is a need for advanced methods to analyze multiple parameters at the same time. This application note presents two methods: HILIC for polar medium compounds, and protein A affinity chromatography for product titer determination. Both methods were run on a single 1260 Infinity II Bio-Inert LC System coupled to an LC/MSD iQ with an automated column selection valve.

## Experimental

### Equipment

The 1260 Infinity II Bio-Inert LC System coupled to the LC/MSD iQ comprised the following modules:

- Agilent 1260 Infinity II Bio-Inert pump (G5654A)
- Agilent 1260 Infinity II Bio-Inert multisampler (G5668A) equipped with Agilent InfinityLab sample thermostat (G5668A#101) and the multiwash option (G5668A#112)
- Agilent 1260 Infinity II multicolumn thermostat (G7116A) equipped with a bio-inert 4-position/10-port column selection valve (G5639A) and divider assembly for use with different temperature zones (G7116-60006)
- Agilent 1260 Infinity II diode array detector WR (G7115A) with bio-inert standard flow cell, 10 mm (G4212-60007)
- Agilent InfinityLab LC/MSD iQ (G6160AA)
- Agilent InfinityLab Flex Bench MS (G6015B)

### Software

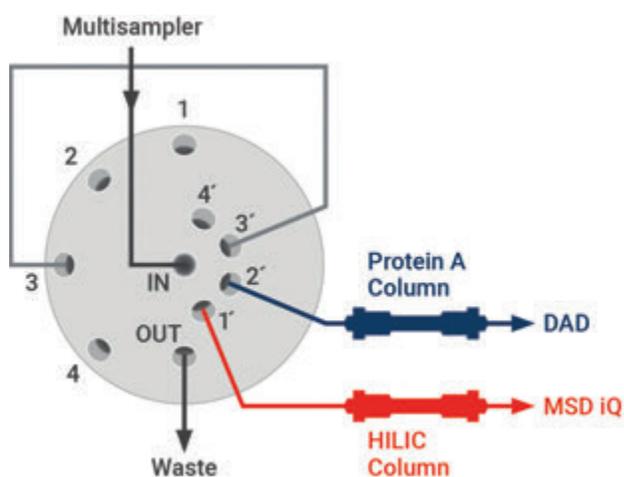
Agilent OpenLab CDS Version 2.4

### Columns

- Agilent InfinityLab Poroshell 120 HILIC-OH5, 2.1 × 100 mm, 2.7 μm (p/n 685775-601)
- Agilent Bio-Monolith protein A, 4.95 × 5.2 mm (p/n 5069-3639)

### Valve configuration

A bio-inert 4-position/10-port column selection valve was installed and configured according to Figure 1, to achieve spent media analysis and mAb titer analysis on a single LC system without reconfiguration between runs. The additional bypass capillary on position 3 enables the flushing of the LC system upstream of the column selection valve, avoiding contact between the mobile phases used for the two methods and potential precipitation of salts.



**Figure 1.** Schematic presentation of the configured bio-inert 4-position/10-port column selection valve.

## Chemicals

LC-grade acetonitrile was 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). D-glucose, L-glutamic acid, L-glutamine, L-lactic acid, and L-alanyl-L-glutamine were purchased from Sigma-Aldrich (Steinheim, Germany). Additionally, a premixed amino acid standard was used (p/n 5061-3330, 1 nmol/µL). Ammonium formate and formic acid were obtained from VWR (Darmstadt, Germany).

## Standards and sample preparation

Stock solutions of all analytes were prepared in water as reference standards with a concentration of 10 mM. Aqueous standards or cultivation supernatants (provided by the Institute of Biochemical Engineering, University of Stuttgart) were either injected directly after centrifugation (12.100 × g, 2 minutes) for titer analysis via protein A chromatography or diluted 1:10 with water, centrifuged, and adjusted to 60% (v/v) acetonitrile and 30 mM ammonium formate (pH 3.0) for HILIC chromatography. The sample predilution with organic solvent and buffer was required to improve the chromatographic performance of the media compounds.

## Cultivation

Bioreactor cultivations of an IgG<sub>1</sub> monoclonal antibody (anti-IL-8) producing CHO DP12 cell line were conducted in batch mode with a starting volume of 1.0 L. The medium was chemically defined (TC42, Xell AG, Germany), comprising L-glutamine, L-alanyl-L-glutamine, D-glucose, growth factors, and various other amino acids. Samples were taken daily and filtered through a 0.2 µm PES syringe filter. Resulting supernatants were stored at -70 °C until analysis.

**Table 1.** HILIC method for spent media analysis.

Parameter	Value
Column	Agilent InfinityLab Poroshell 120 HILIC-OH5, 2.1 × 100 mm, 2.7 µm
Solvent	A) 85% acetonitrile / 5% 2-propanol / 10% water + 30 mM ammonium formate, pH 3 B) 10% acetonitrile / 5% 2-propanol / 85% water + 30 mM ammonium formate, pH 3
Gradient	0.00 min – 100% A/0% B 1.00 min – 100% A/0% B 11.00 min – 90% A/10% B 12.00 min – 0% A/100% B 12.01 min – 100% A/0% B 20.00 min – 100% A/0% B
Flow rate	0.600 mL/min
Temperature	20 °C
MS Detection	Auto Acquire mode / SIM (see Table 3)
Injection	Injection volume: 1 µL Sample temperature: 4 °C Multiwash: 5 s in 90% acetonitrile / 10% water (S1, needle wash)

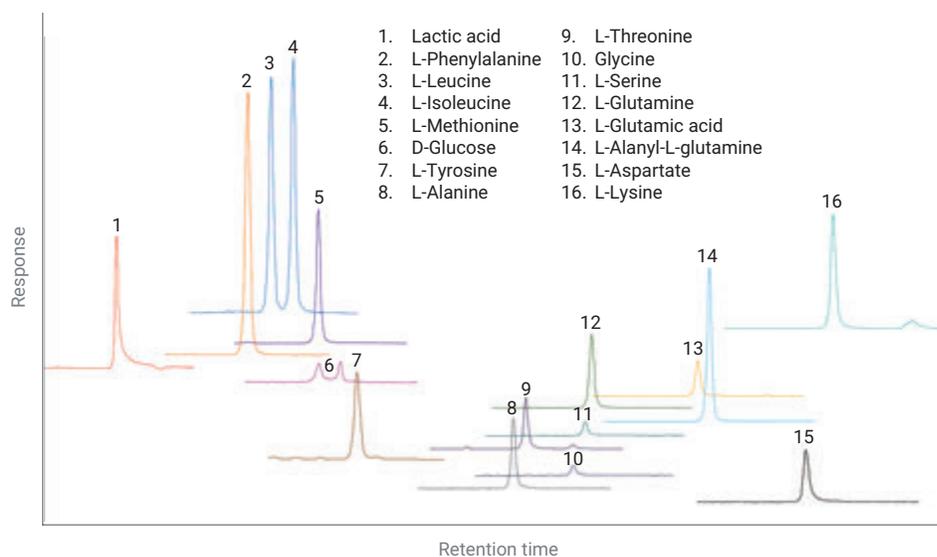
**Table 2.** Protein A method for mAb titer analysis.

Parameter	Value
Column	Agilent Bio-Monolith protein A, 4.95 × 5.2 mm
Solvent	C) 50 mM sodium phosphate buffer pH 7.4 D) 500 mM acetic acid, pH 2.6
Gradient	0.00 min – 100% C/0% D 0.50 min – 100% C/0% D 0.60 min – 0% C/100% D 1.70 min – 0% C/100% D 1.80 min – 100% C/0% D 3.5 min – 100% C/0% D
Flow Rate	1 mL/min
Temperature	30 °C
UV detection	280 nm/4 nm, reference 360 nm/100 nm, 20 Hz
Injection	Injection volume: 50 µL Sample temperature: 4 °C Multiwash: 5 s in 100% water (S2, needle wash)

## Results and discussion

Chemically defined cell culture media for the cultivation of CHO cells comprise several polar chemicals that can be analyzed without prior derivatization on HILIC columns. Since most of these compounds lack a chromophore, standard UV detection is not suitable. In this case, easy-to-use mass detection, as offered by the LC/MSD iQ, provides increased specificity and selectivity. By using the Auto Acquire mode, ion source parameters and fragmentor voltages were automatically adjusted based on the configured LC flow rate and ion mass of the compound. Only appropriate SIM channels were manually configured. For separation, an InfinityLab Poroshell 120 HILIC-OH5 column was used with ammonium formate as buffer at low pH (Table 1). In addition, 5% (v/v) 2-propanol was added as an organic modifier to mobile phases A and B to optimize peak shapes in the HILIC separation. Figure 2 shows the separation of 16 polar compounds typically present in a cell culture medium comprising organic acids, amino acids, and dipeptides.

To demonstrate the ability of the LC/MSD iQ for external quantification, a 10-point calibration curve for every compound with the following levels was prepared: 1, 2, 5, 10, 20, 50, 75, 100, 150, 200  $\mu\text{M}$ . Additionally, the limit of detection (LOD, S/N = 3) was determined with an automated signal-to-noise (S/N) calculation (P2P) in OpenLab CDS for the average of the three lowest levels for every compound. Table 3 shows good linearity ( $R^2 > 0.99$ ) and LODs below one pmol on-column for most analytes, demonstrating that the LC/MSD iQ is a great choice for user-friendly quantification with a small lab footprint.<sup>3</sup>



**Figure 2.** Chromatograms of cell culture medium compounds, analyzed as standards (200  $\mu\text{M}$ ) with the HILIC method and Agilent InfinityLab LC/MSD iQ as a detector.

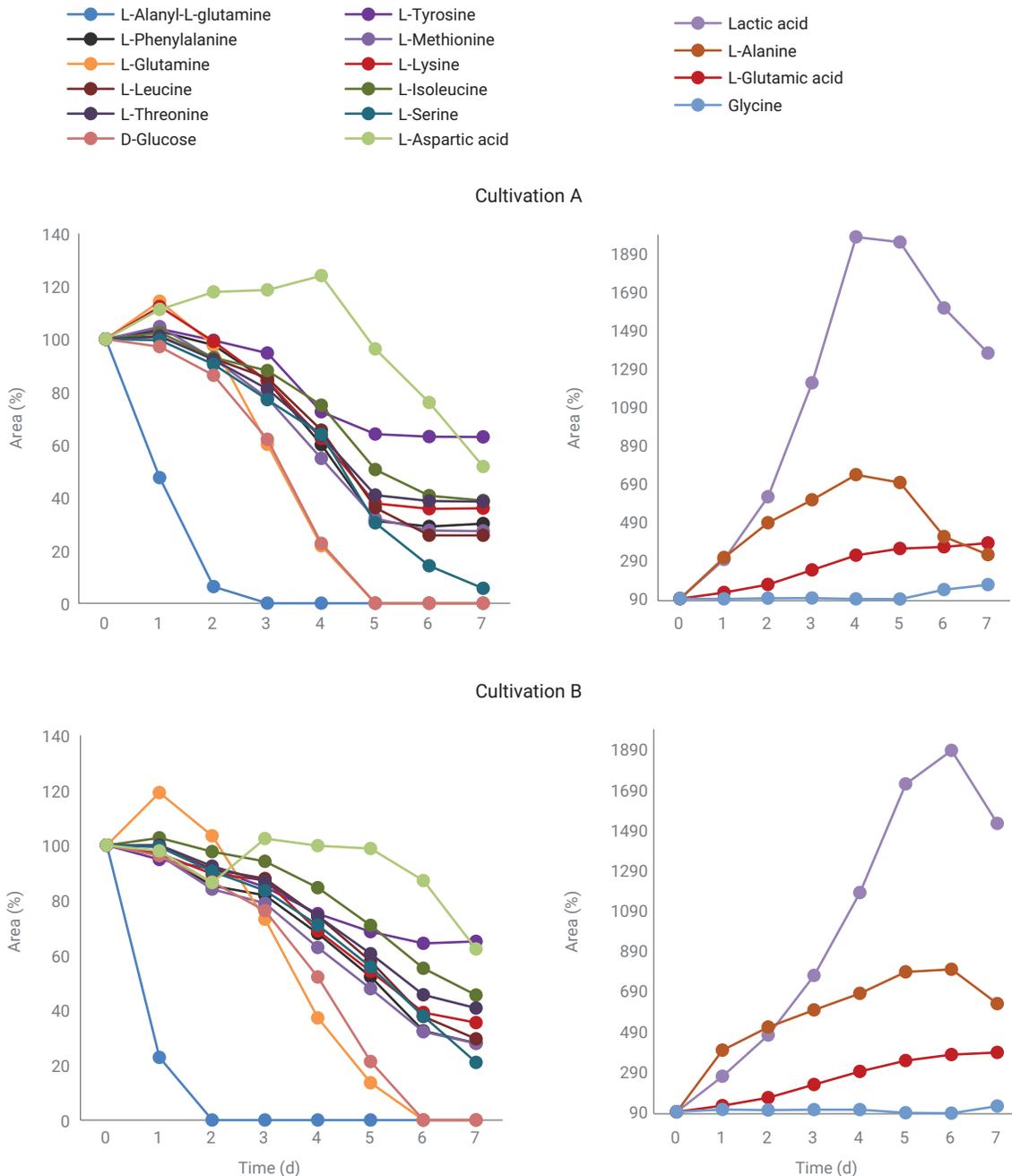
**Table 3.** MS and quantification parameters for each compound standard analyzed in the HILIC spent media analysis.

Compounds	SIM (m/z)/ Polarity	Fragmentor (V)	Retention Time (min)	Calibration Range ( $\mu\text{M}$ )	$R^2$	LOD (pmol On-Column)
Lactic acid	89 / -	90	1.734	1 to 200	0.9979	0.63
L-Phenylalanine	166 / +	100	3.611	1 to 200	0.9956	0.41
L-Leucine	132 / +	90	3.951	1 to 200	0.9964	0.86
L-Isoleucine	132 / +	90	4.274	1 to 200	0.9964	0.67
L-Methionine	150 / +	100	4.615	1 to 200	0.9966	0.23
D-Glucose	179 / -	100	4.917	20 to 200	0.9934	12.24
L-Tyrosine	182 / +	100	5.134	2 to 200	0.9997	1.09
L-Alanine	90 / +	90	7.358	1 to 200	0.9980	0.88
L-Threonine	120 / +	90	7.534	1 to 200	0.9984	0.90
Glycine	76 / +	90	8.218	5 to 200	0.9991	3.72
L-Serine	106 / +	90	8.387	5 to 200	0.9982	2.80
L-Glutamine	147 / +	90	8.512	2 to 200	0.9989	1.72
L-Glutamic acid	148 / +	90	10.100	2 to 200	0.9996	1.46
L-Alanyl-L-glutamine	218 / +	100	10.158	1 to 200	0.9952	0.54
L-Aspartate	132 / -	90	11.537	2 to 200	0.9958	1.19
L-Lysine	147 / +	90	11.969	1 to 150	0.9901	0.29

Two CHO cell cultivations (A+B) were performed, and bioreactors were sampled daily to show the applicability of the latter HILIC method for spent media analysis. The dynamic profiles of the nutrients are shown in Figure 3. On the left side, all compounds are depicted as having decreasing concentrations during the cultivation. Compounds with

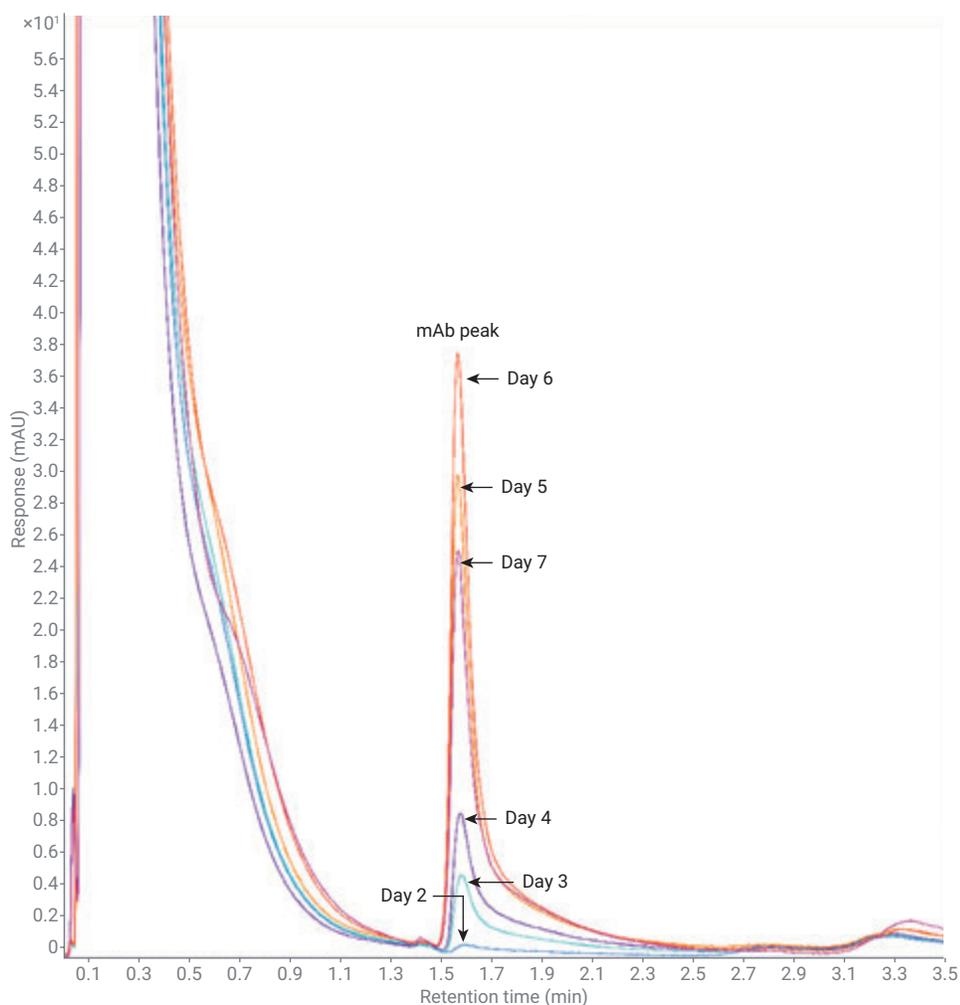
increasing concentrations are shown on the right side of the diagram. Overall, the medium components indicate a typical behavior, for example, the consumption of D-glucose (carbon source) and L-glutamine/L-alanyl-L-glutamine (nitrogen source) or the formation of lactic acid as a side product during overflow metabolism. Slight

differences in the compound formation or consumption rate demonstrate the different performances of CHO cell cultivations (A versus B). By applying this HILIC method during the cultivation, in-process measures could be taken to control the process and optimize product yield at the end of cultivation.



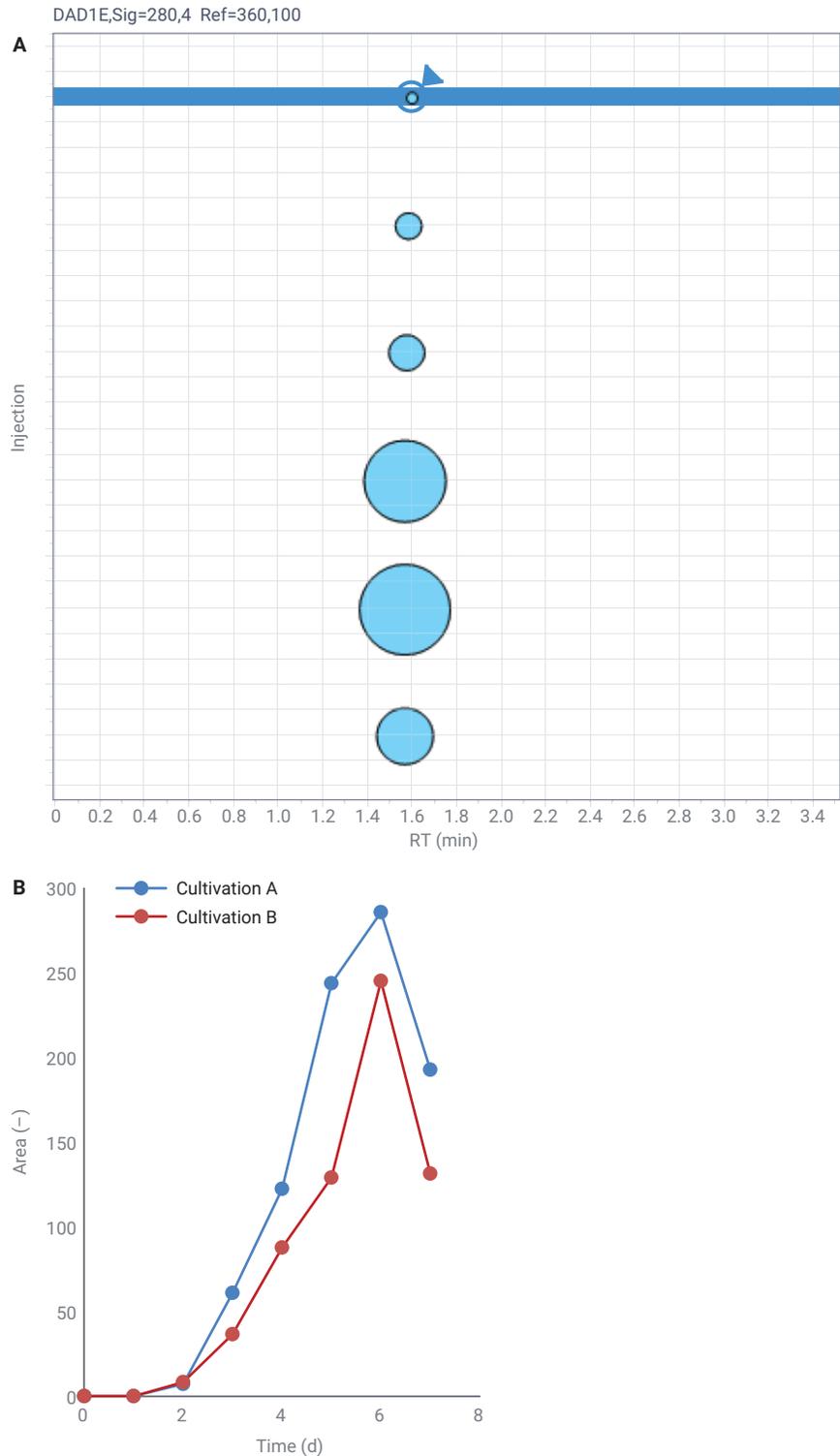
**Figure 3.** Profiles of HILIC-analyzed media compounds during cell culture cultivations.

To further monitor process variables, protein A affinity chromatography was applied subsequently on the same 1260 Infinity II Bio-Inert LC System, which features a fully bio-inert flow path, especially suited for sticky biomolecules and metabolites. Using all four available solvent channels (A + B for HILIC, C + D for protein A), both methods could be run without manual solvent switching. Additionally, both columns were installed at the same time, controlled at different temperatures, and could be switched by an internal column selection valve, further decreasing manual operations. The developed and optimized protein A method is depicted in Table 2. Figure 4 shows UV chromatograms for the protein A method of cultivation A over six cultivation days. The mAb titer reached a maximum at day six and decreased afterward, probably due to thermal or enzymatic degradation of the Fc region of the mAb, which binds to protein A.



**Figure 4.** Chromatograms of supernatant samples of cultivation A over the course of six fermentation days, analyzed by the protein A method coupled to the Agilent 1260 Infinity II diode array detector (at 280 nm).

After the integration of mAb peaks, OpenLab offers a feature called Peak Explorer. Figure 5A shows a snapshot of the Peak Explorer view, where the Y-axis shows the different injections and the X-axis the corresponding retention times. The area of the integrated mAb peak is depicted as a circle, where the diameter of the circle is proportional to the amount of mAb. This Peak Explorer view gives a quick qualitative and straightforward display of the dynamic mAb production. When comparing the dynamic mAb accumulation of cultivation A and B, significant differences in the increase of mAb became obvious (Figure 5B), which could again be a signal for in-process adjustments. Combining both methods presents an excellent toolbox for employing at-line process analysis in a production or process development environment.



**Figure 5.** A snapshot of the Peak Explorer view for cultivation A in Agilent OpenLab CDS (A) and mAb production during the cultivation period (B).

## Conclusion

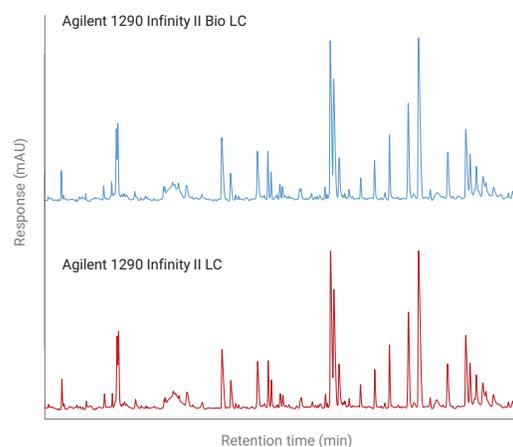
Process analytical technology (PAT) is a powerful tool to adjust and improve bioprocess parameters such as growth rate, product yield, and product quality during the production process. As a prerequisite, we showed the application of HPLC measurements as a means for process control in this application note. The presented HILIC analysis with the LC/MSD iQ as detector showed excellent linearity, sensitivity, and selectivity of key cell culture nutrients with the easy-to-use Auto Acquire mode. By combining spent media analysis and protein A affinity chromatography on a 1260 Infinity II Bio-Inert LC coupled to an LC/MSD IQ with automated column switching, manual operations were significantly reduced. Paired with OpenLab CDS 2 software, which can be fully qualified and is available with all necessary services to meet the requirements of the FDA regarding 21CFR Part 11, this at-line HPLC solution is fully suited to be part of a modern QbD environment.

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3. Feith, A. *et al.* HILIC-Enabled <sup>13</sup>C Metabolomics Strategies: Comparing Quantitative Precision and Spectral Accuracy of QTOF High- and QQQ Low-Resolution Mass Spectrometry. *Metabolites* **2019**, *9*, 63.

# Seamless Method Transfer to the Agilent 1290 Infinity II Bio LC System

Peptide-mapping analysis shows excellent performance and high method compatibility compared to the Agilent 1290 Infinity II LC System

**BIO**

## Author

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## Abstract

Peptide mapping requires reliable and robust methods with high precision for analyzing the primary structure and post translational modifications (PTMs) of biopharmaceuticals. However, method transferability and compatibility can be an issue for validated methods. This application note shows that method transfer can be easy and convenient with the new Agilent 1290 Infinity II Bio LC System. Building on the excellent average relative retention time deviation of 0.039% for 12 selected peptides, it was discovered that the retention times only deviated by 0.17% between the 1290 Infinity II Bio LC System and the Agilent 1290 Infinity II LC System. By combining the LC systems with the Agilent 6545XT AdvanceBio LC/Q-TOF, additional comparative statistical analysis of peak abundances revealed no significant differences between both systems, rendering the new 1290 Infinity II Bio LC the ideal choice for UV- or MS-based peptide-mapping workflows.

## Introduction

Method transfer and compatibility from one instrument to another are important for laboratories across different industries.<sup>1</sup> Especially in the biopharmaceutical industry, instrument-to-instrument method transfer is highly important for validated methods. To demonstrate the seamless method transfer from the 1290 Infinity II LC to the 1290 Infinity II Bio LC, the peptide-mapping workflow was chosen because of its considerable relevance in the evaluation of biological products as described in ICH Guideline Q6B.<sup>2</sup> Employing a tryptic digest of the NISTmAb, this application note will show that method transfer can be straightforward thanks to the 1290 Infinity II Bio LC.

## Experimental

### Equipment

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

Agilent 1290 Infinity II Bio LC:

- 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)

Agilent 1290 Infinity II LC:

- Agilent 1290 Infinity II High-Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116A) equipped with a Standard Flow Quick Connect Heat Exchanger (G7116-60015) and two Agilent Thermal Equilibration Devices (G7116-60013)
- Agilent 1290 Infinity II Variable Wavelength Detector (VWD) (G7114B), equipped with a 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 Mass Profiler (B.10.00)

### Columns

- 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 of 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.

## Results and discussion

To show the excellent performance and method transfer between the 1290 Infinity II Bio LC and the 1290 Infinity II LC, a tryptic digest of the NISTmAb was analyzed with UV and MS detection. Both systems were equipped with capillaries of the same length and diameters to have similar extra column volumes. However, the 1290 Infinity II Bio LC featured a completely iron-free flow path especially suited for sticky biomolecules. For both analyses, the same ZORBAX RRHD Eclipse Plus column and method parameters were used (Table 1). Figure 1 shows the chromatograms of the peptide maps acquired by both systems. Excellent similarities between the peptide patterns are visible, with almost no detectable differences.

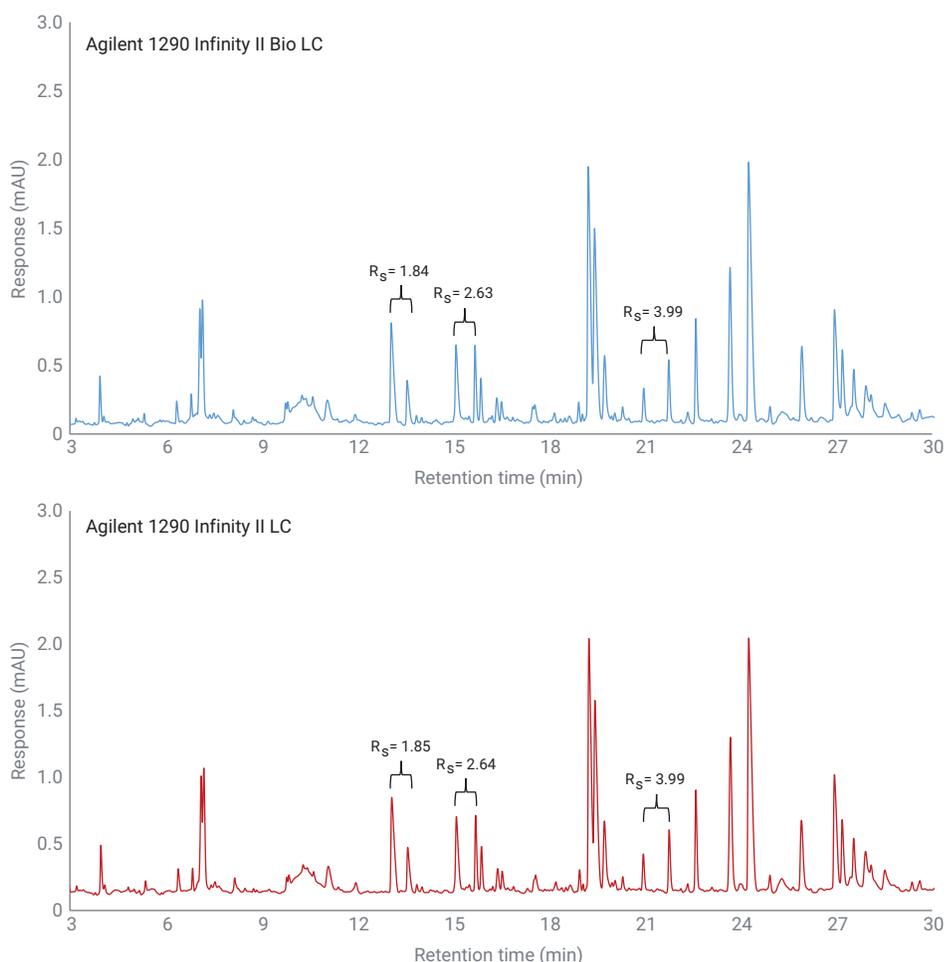
**Table 1.** Peptide-mapping method for the Agilent 1290 Infinity II LC and Bio LC.

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 2
Injection	Injection volume: 15 μL Sample temperature: 4 °C Wash: 3 s with water (flush port)

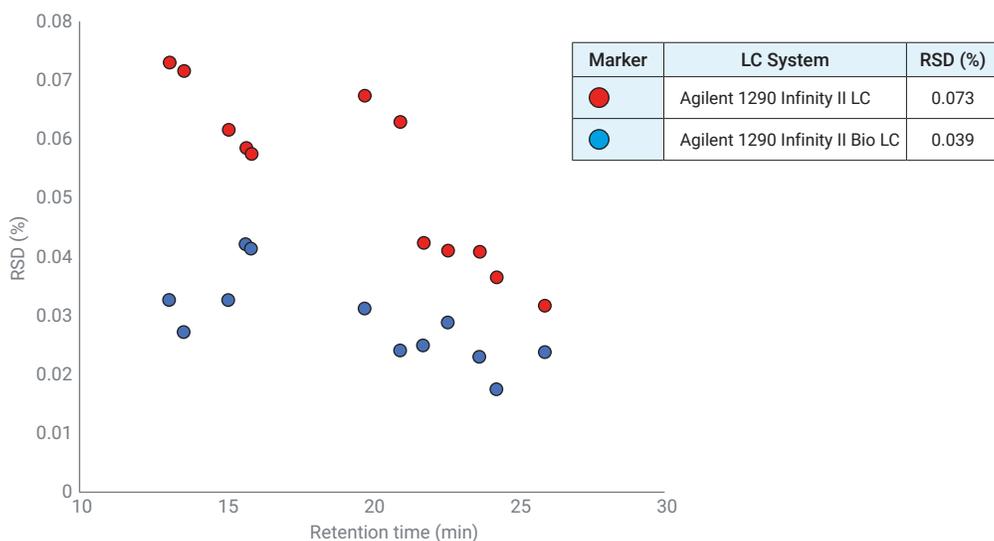
**Table 2.** Source and MS parameters for the All Ions MS/MS analysis of peptides.

Parameter	Value
Instrument	Agilent 6545XT AdvanceBio LC/Q-TOF
Gas Temperature	300 °C
Drying Gas Flow	13 L/min
Nebulizer	40 psig
Sheath Gas Temperature	350 °C
Sheath Gas Flow	12 L/min
Vcap	4,000 V
Nozzle Voltage	500 V
Fragmentor	175 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Acquisition Mode	Positive, extended dynamic range (2 GHz)
Mass Range	<i>m/z</i> 100 to 1,700
Acquisition Rate	6 spectra/sec
Collision Energy	All ions MS/MS—0 V, 10 V, 25 V

For better evaluation, three generic resolution ( $R_s$ ) values were calculated for both separations (Figure 1) and also showed exceptionally good comparability. To analyze the performance of the 1290 Infinity II Bio LC and 1290 Infinity II LC regarding retention time precision, 12 peptides were chosen, and the corresponding relative standard deviations (RSD) of the retention times were calculated based on 10 consecutive injections. Figure 2 depicts that all RSD values, irrespective of the system, are below 0.1%, showcasing the excellent performance of the Agilent 1290 Infinity II Bio High-Speed Pump and 1290 Infinity II High-Speed Pump. The average RSD value of the 12 peptides even gets as low as 0.039% for the 1290 Infinity II Bio LC, rendering this system an excellent choice for robust and reliable peptide mapping. However, besides high performance, method compatibility between different LC systems is also very important for numerous labs.



**Figure 1.** Chromatograms of a tryptic digest of the NISTmAb separated by the Agilent 1290 Infinity II Bio LC and the Agilent 1290 Infinity II Bio LC with the same method (Table 1).

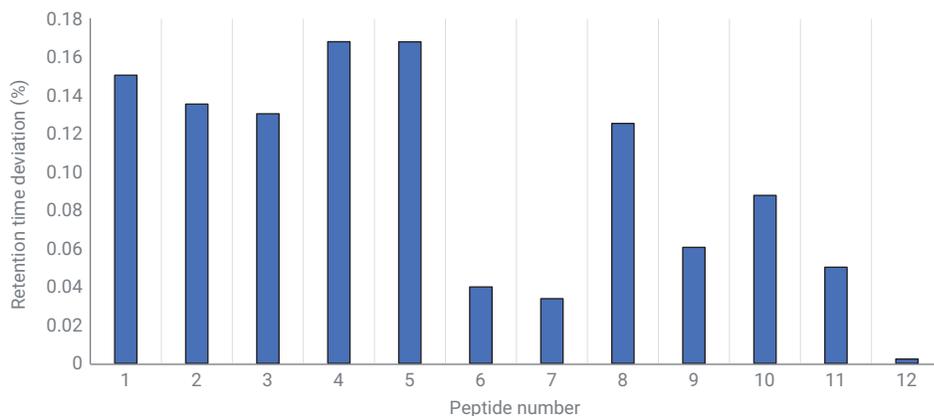


**Figure 2.** Relative retention time precision (RSD) values for the Agilent 1290 Infinity II Bio LC and the Agilent 1290 Infinity II LC.

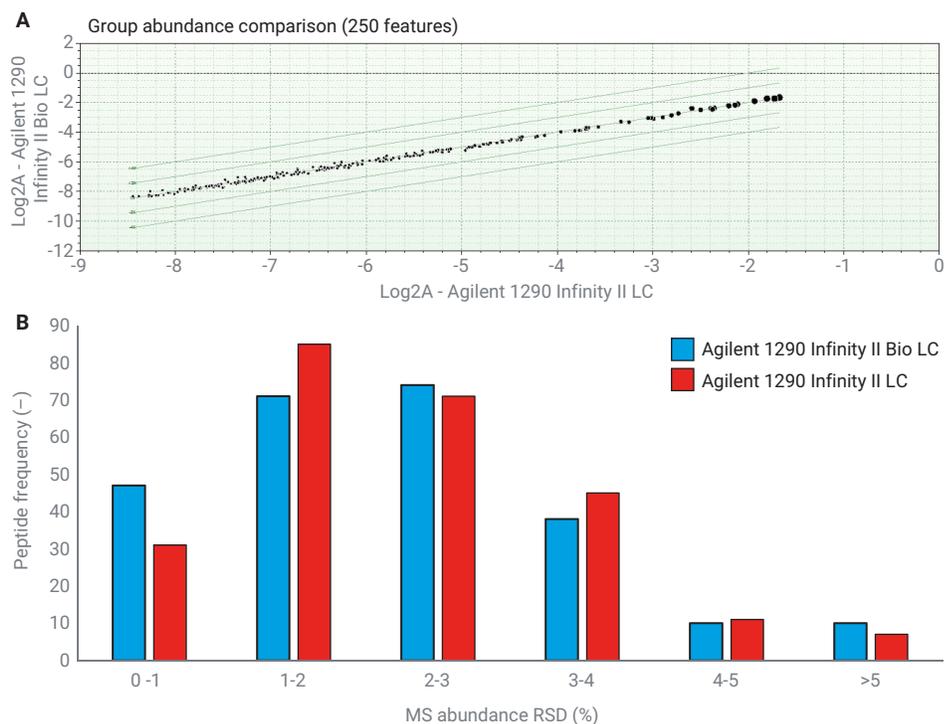
Absolute retention times need to be in certain windows to identify analytes in a validated or compliance environment. By determining the average retention times of the 12 peptides for both LC systems and calculating the deviation of the retention times between the 1290 Infinity II Bio LC and 1290 Infinity II LC, the performances were evaluated. Average peptide retention times are depicted in the table of Figure 3 and corresponding deviations are shown as bar plots. Minimal deviations of up to 0.17% between the LC systems were calculated, showing seamless method transfer between the 1290 Infinity II Bio LC and 1290 Infinity II LC.

To further investigate the method compatibility, both systems were coupled to the Agilent 6545XT AdvanceBio LC/Q-TOF. In an untargeted approach, the MS detector was used in All Ions mode (Table 2), periodically fragmenting all precursor ions in the collision cell. These information-rich data sets were then evaluated with the Agilent MassHunter Mass Profiler (B.10.00) software for both LC systems to get a holistic view of the differences in the abundance of identified peaks. Ten consecutive injections of a tryptic digest of the NISTmAb on both LC systems were the basis for subsequent statistical analysis. The 250 most abundant peaks were evaluated by correlation analysis, and the corresponding log-fold changes are depicted in Figure 4A. If a peak does not differ in both systems, it will cluster around the 1x line in Figure 4A, signaling no significant difference in the peak area. However, if there is a peak with a two-times higher abundance in one system, it would be located around the 2x line. Looking at the graphical data results, it becomes clear that there are no significant differences for most peaks. Up to 75% of the peaks varied with 10% or less in abundance. Even more

LC System	Average Peptide Retention Times (min)											
	1	2	3	4	5	6	7	8	9	10	11	12
Agilent 1290 Infinity II LC	13.082	13.577	15.104	15.704	15.887	19.751	20.968	21.769	22.599	23.684	24.261	25.907
Agilent 1290 Infinity II Bio LC	13.062	13.559	15.084	15.677	15.860	19.743	20.961	21.742	22.585	23.663	24.249	25.907



**Figure 3.** Average retention times for the 12 chosen peptides and their deviations between the two LC systems.



**Figure 4.** (A) Statistical correlation analysis based on 10 consecutive injections. Log fold values are depicted as black dots for the 250 highest abundance peaks identified by Agilent MassHunter Mass Profiler (B.10.00). (B) Histogram for the RSD values of the peptide abundances for the Agilent 1290 Infinity II Bio LC and 1290 Infinity II LC.

strikingly, the RSD for the abundances over 10 injections were nearly the same for the 1290 Infinity II Bio LC and 1290 Infinity II LC (Figure 4B). Over 90% of the peptide peaks had an area RSD value of 4% or less.

## Conclusion

Method transfer can sometimes be a laborious and difficult process for many labs when configuring and installing a new LC system. This application note showed that this is not the case for the Agilent 1290 Infinity II Bio LC. By running the same NISTmAb peptide-mapping method on the 1290 Infinity II Bio LC and 1290 Infinity II LC, it was shown that the method could be seamlessly transferred with retention time deviations of only up to 0.17% between the systems. Thanks to the 1290 Infinity II Bio High-Speed Pump, the average relative retention time deviations after 10 injections also showed an excellent value with 0.039%. By coupling both systems with the 6545XT AdvanceBio LC/Q-TOF, a comprehensive statistical analysis of peak abundances showed no significant differences and excellent average RSD of 2.8%. Combining these findings, it is clear that efficient and convenient method transfer between the 1290 Infinity II Bio LC and 1290 Infinity II LC can easily be achieved. The 1290 Infinity II Bio LC is therefore the ideal choice for peptide-mapping workflows regardless of the detection method, with the benefit of an iron-free flow path.

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[www.agilent.com/chem](http://www.agilent.com/chem)

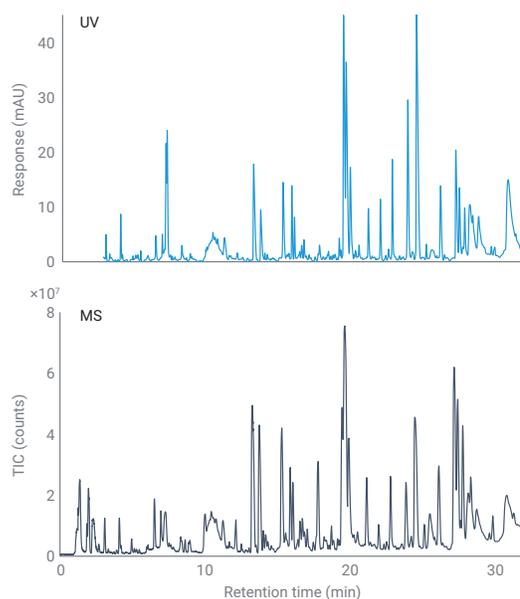
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## Robust and Reliable Peptide Mapping

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

**BIO**

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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.<sup>1</sup> By using LC/MS or UV, changes in the peptide map—for example, increased oxidation or deamidation,<sup>2</sup> the appearance of new sequence variants,<sup>3</sup> or changes in the glycan composition<sup>4</sup>—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  $\mu$ L, 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  $\times$  250 mm, 2.7  $\mu$ m (part number 651750-902)
- Agilent AdvanceBio Peptide Mapping Fast Guards, 2.1  $\times$  5 mm, 2.7  $\mu$ m (part number 851725-911)
- Agilent ZORBAX RRHD Eclipse Plus C18, 2.1  $\times$  150 mm, 1.8  $\mu$ m (part number 959759-902)
- Agilent ZORBAX RRHD Eclipse Plus C18 Fast Guards, 2.1  $\times$  5 mm, 1.8  $\mu$ m (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  $\mu$ m 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  $\mu$ L ammonium bicarbonate (100 mM) was denatured and reduced by the addition of 2  $\mu$ L of *tris*(2-carboxyethyl)phosphine (TCEP, 200 mM) and incubated at 60  $^{\circ}$ C for 1 hour. After the alkylation with 4  $\mu$ L of 2-iodoacetamide (IAM, 200 mM, 1 hour at RT), quenching of excess IAM with 2  $\mu$ L 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  $^{\circ}$ C, the pH of the resulting suspension was decreased below pH 4 by the addition of 2  $\mu$ L of formic acid.

**Table 1.** Method A: Comprehensive NISTmAb peptide-mapping method adapted from Mouchahoir & Schiel, 2018.<sup>5</sup>

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)

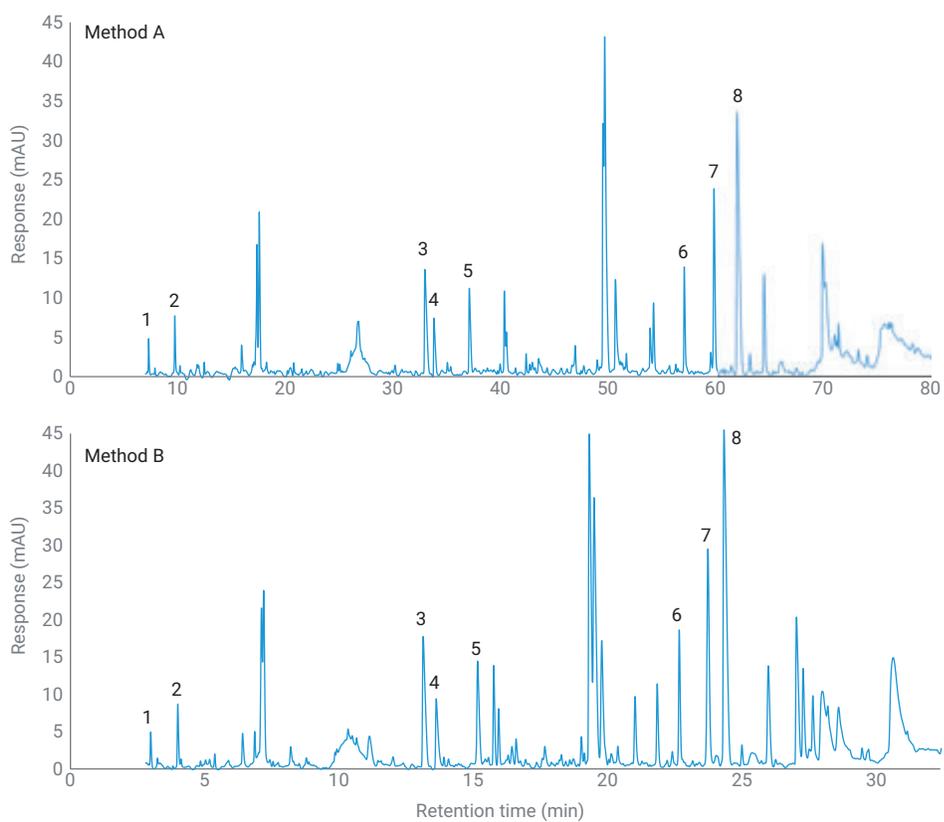
**Table 3.** Source and MS parameters for the iterative MS/MS analysis of peptides.

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	<i>m/z</i> 100 to 1,700
Acquisition Rate	8 spectra/sec
Auto MS/MS Range	<i>m/z</i> 50 to 1,700
Isolation Width	Narrow (~ <i>m/z</i> 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

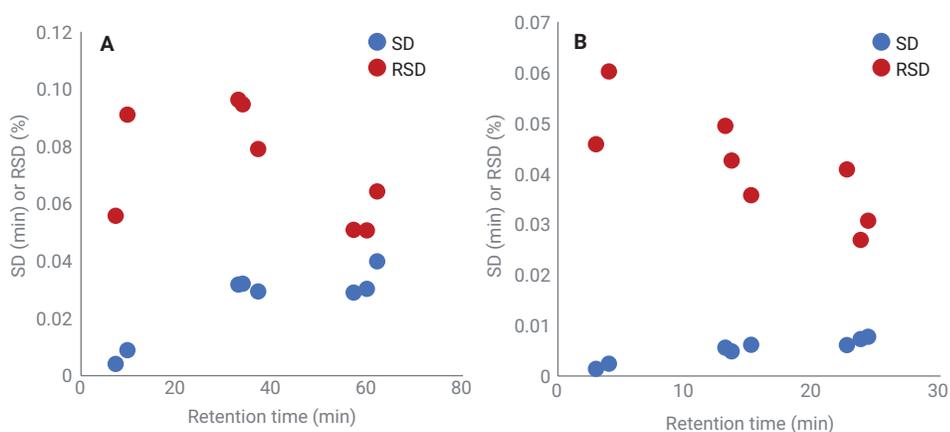
## Results and discussion

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.<sup>5</sup> 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  $\mu\text{m}$  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)	$\emptyset$ SD (min)	$\emptyset$ RSD (%)	$P_{C,As}$ (-)
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.

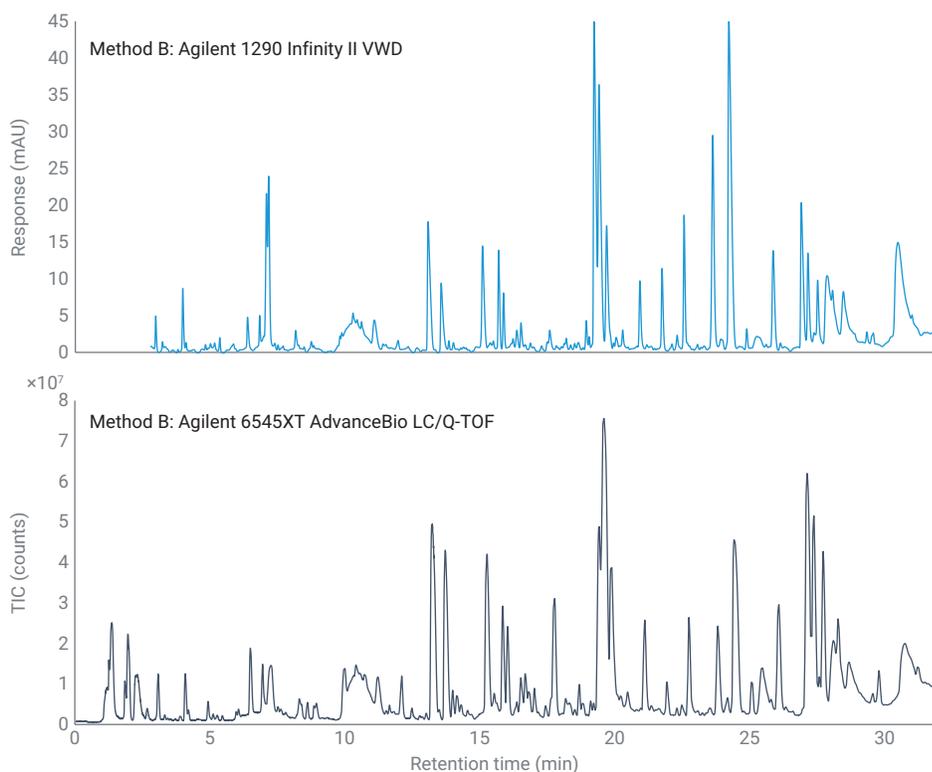
Methods A and B both show relative retention time deviations below 0.1%, displaying the exceptional performance of the 1290 Infinity II Bio High-Speed Pump even at very shallow gradient slopes of 0.39 and 0.98% B/min, respectively. To evaluate the comprehensive separation character of the LC methods,  $4\sigma$  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)<sup>6</sup> 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.



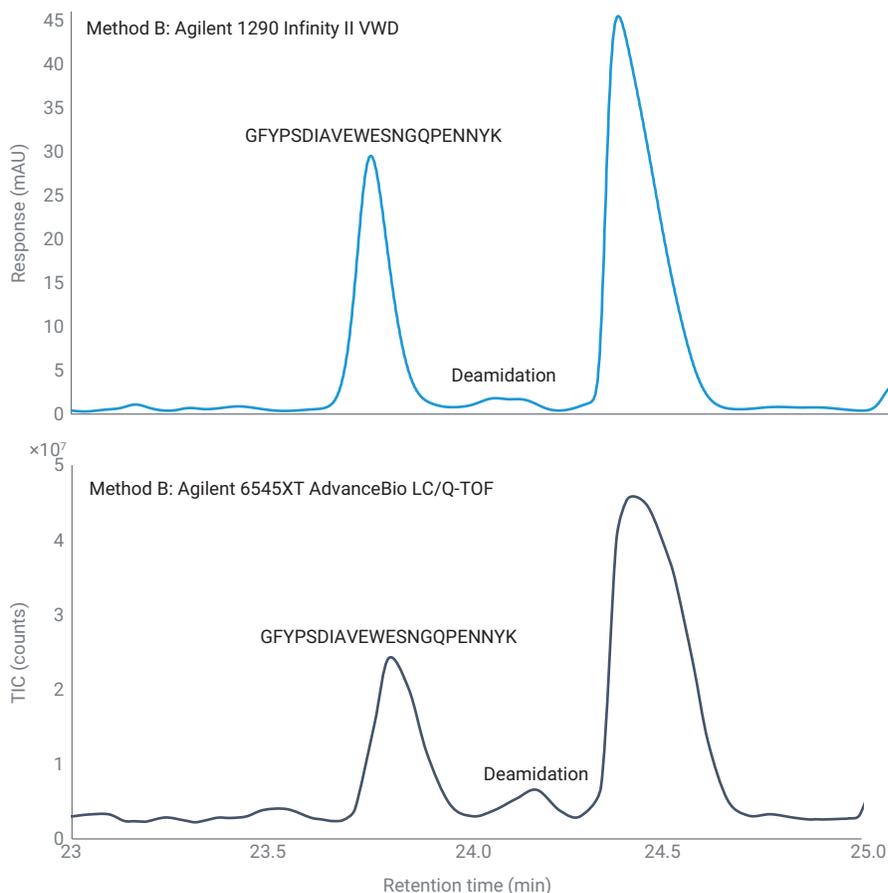
**Figure 3.** Chromatograms of a tryptic digest of the NISTmAb detected in sequence with the Agilent 1290 Infinity II VWD equipped with the biocompatible micro flow cell (upper) and the Agilent 6545XT AdvanceBio LC/Q-TOF (lower).

## 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.

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**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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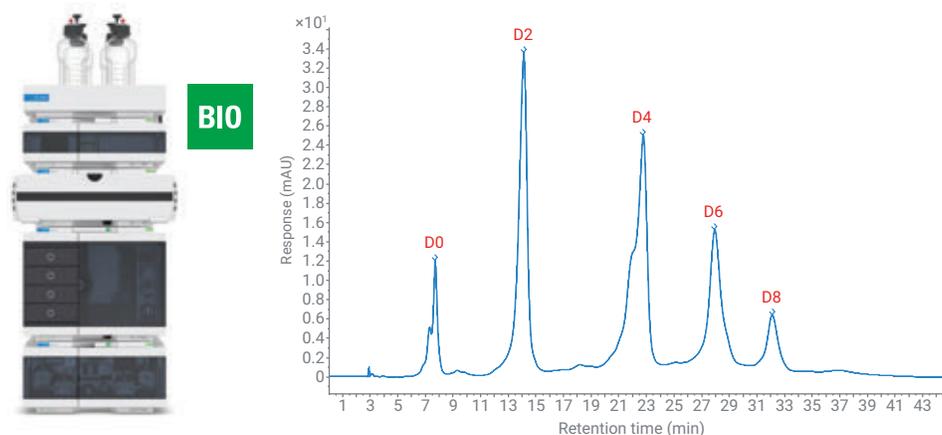
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## High Salt—High Reproducibility

Analysis of antibody drug conjugates using hydrophobic interaction chromatography with the Agilent 1290 Infinity II Bio LC System



### Author

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### Abstract

The determination of the drug-to-antibody ratio (DAR) is typically performed using hydrophobic interaction chromatography (HIC). The eluents for this mild, non-denaturing analysis method contain high concentrations of corrosive salts, which challenge the liquid chromatography (LC) system.

The Agilent 1290 Infinity II Bio LC System including High-Speed Pump, with its completely iron-free flow path, is optimally suited for the conditions used in biochromatography—avoiding potential corrosive damage to the system. This application note demonstrates the DAR determination of brentuximab vedotin using HIC. The DAR was calculated to 3.7 drug molecules per antibody. Excellent reproducibility was found, demonstrating that the 1290 Infinity II Bio LC belongs to the next generation of Agilent high-end liquid chromatography systems for the highest confidence in generated data. “Blank subtraction”, as a software feature of Agilent OpenLab CDS, removes drifting baselines due to less pure ammonium sulfate, enabling smooth integration.

## Introduction

Antibody drug conjugates (ADCs) are monoclonal antibodies (mAbs) to which a cytotoxic small molecule drug is chemically linked.<sup>1</sup> Compared to their corresponding antibodies, the structure is more complex and heterogeneous.

Cysteine-linked ADCs<sup>2</sup> (such as brentuximab vedotin, Adcetris by Takeda) has the small molecule attached to the free thiol groups of the partially reduced mAb.<sup>3,4</sup> The number of free sulfhydryl groups limits the number of defined positions for the drug to be conjugated, resulting in a mixture of zero, two, four, six, and eight drugs per antibody. The average number of drugs conjugated to the mAb is one of the most important quality attributes of an ADC because it can directly affect safety and efficacy. The DAR determines the amount of payload that can be delivered to the desired tissue.<sup>5</sup>

HIC is the reference technique to separate cysteine-linked ADC molecules loaded with different numbers of drugs per antibody.<sup>6</sup> The relative hydrophobicity increases with the drug load of the ADC because the small molecules attached to the mAb are often relatively hydrophobic. Therefore, HIC is perfectly suited to monitor the DAR.

HIC is a non-denaturing analysis technique maintaining the native protein structure. It is typically performed at neutral pH, separating the proteins with a gradient from high to low salt concentration. The separation principle is the same as found in protein salting-out experiments.<sup>6</sup> In the high-concentration salt buffer used in mobile phase A, the proteins lose their hydration shell and are retained on the hydrophobic surface of the stationary phase. Mobile phase B is usually the same buffer (mostly phosphate) without added salt. With an increasing amount of mobile phase B in

the gradient, the proteins re-assemble the water shell and are eluted from the column. The addition of a small amount of organic solvent such as isopropyl alcohol can also help to elute the proteins from the column.

The 1290 Infinity II Bio LC is the next generation of Agilent high-end liquid chromatography systems, specially designed for conditions used in biochromatography: high salt concentrations such as 2 M NaCl,<sup>7</sup> up to 8 M urea, and high- and low-pH solvents such as 0.5 M NaOH or 0.5 M HCl. The sample flow path is completely free of stainless steel (SST) or iron; all capillaries and fittings throughout the multisampler, multicolumn thermostat, and detectors are built of MP35N, a nickel-cobalt alloy. With this material, potential corrosion from high salt-containing buffers is reduced and protein modifications caused by the presence of iron ions (e.g., oxidation, protein complex formation) can be avoided.

This application note presents the analysis of brentuximab vedotin with HIC for the determination of DAR, evaluating the precision of retention time and area. In addition, the advantages of the software feature "Blank Subtraction" in the processing method of OpenLab 2 are demonstrated to filter drifting baselines.

## 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) with standard flow biocompatible heat exchanger

- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2  $\mu$ L

### Software

Agilent OpenLab CDS Version 2.5

### Columns

Agilent AdvanceBio HIC column, 3.5  $\mu$ m, 4.6  $\times$  100 mm (p/n 685975-908)

### Chemicals

All solvents were LC grade. Isopropanol was purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22  $\mu$ m membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, and ammonium sulfate were obtained from Sigma-Aldrich (Steinheim, Germany).

### Samples

Brentuximab vedotin (Trade name Adcetris by Takeda, Tokyo, Japan) dissolved in half water, half solvent A (see below) at 100 mg/mL.

**Note:** As Adcetris contains many adjuvants, the concentration mentioned here is not the protein concentration only, but the total concentration of all components of the drug.

### Buffer preparation

**A:** 1.5 M ammonium sulfate in 50 mM phosphate buffer at pH 7

**B:** 50 mM phosphate buffer at pH 7 + 20% isopropanol

For 2 L of 50 mM phosphate buffer, pH 7, 5.84 g of sodium phosphate monobasic monohydrate and 15.47 g of sodium phosphate dibasic heptahydrate were added to an amber-colored 2 L bottle and filled up to 2 L using ultrapure water. The pH value was checked and adjusted,

if necessary, to pH 7. Then, 198.21 g of ammonium sulfate for a total of 1.5 M was added to an empty, amber-colored 1 L bottle and filled up to 1 L using the prepared phosphate buffer (→buffer A). The pH value was checked and adjusted, if necessary, back to pH 7 (the addition of high amounts of salt can change the pH). 200 mL of isopropanol and 800 mL of the prepared 50 mM phosphate buffer, pH 7 was mixed and added to an empty, amber-colored 1 L bottle (→buffer B). Both prepared buffers were filtered using a 0.2-µm membrane filter.

**Note:** The presence of small hydrophobic drug molecules conjugated to the mAb increases the overall hydrophobicity considerably. Consequently, it is necessary to include some organic modifier in the mobile phase (here: 20% isopropanol).

## Method

**Table 1.** Salt gradient chromatographic conditions.

Parameter	Value
Solvent	A) 1.5 M ammonium sulfate in 50 mM phosphate buffer at pH 7 B) 50 mM phosphate buffer at pH 7 + 20% isopropanol
Gradient	0 minutes 30% B, 30 minutes 100% B Stop time: 45 minutes Post-time: 10 minutes
Flow rate	0.400 mL/min
Temperature	25 °C
Detection	280 nm, 10 Hz
Injection	Injection volume: 15 µL Sample temperature: 10 °C Needle wash: 3 s in water

**Note:** The high concentrations of salt used in HIC require a robust LC system, and the completely stainless steel (SST)/iron-free flow path of the 1290 Infinity II Bio LC prevents potential corrosion from high salt-containing buffers. In addition, washing features like seal wash and needle wash help to avoid issues with salt precipitation. However, it is still important to avoid leaving either the LC system or the column in a concentrated salt solution for any length of time.

**Note:** When using concentrated salt solutions as eluents, consider setting corresponding solvent types in the pump method. For example, for solvent A, including 1.5 M ammonium sulfate, use “Ammonium Sulfate 1.5 M” rather than *Generic Aqueous* or *Water* in the solvent selection field in the pump method. High amounts of salts change the compressibility of the solvent, and so using the preconfigured solvent tables enables best pump performance.

## Results and discussion

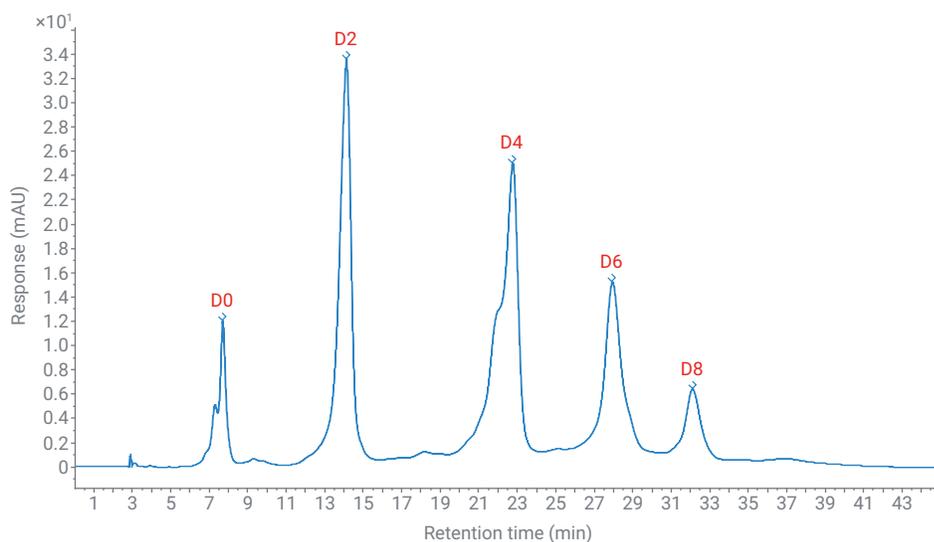
Figure 1 shows the analysis of brentuximab vedotin, revealing five main peaks that correspond to the mAb containing zero, two, four, six, and eight small molecule drugs, respectively. The HIC analysis allowed both the characterization of the distribution of drug-linked species, as well as the determination of the DAR.

Although the interchain disulfide bridges are disrupted and occupied by the conjugated drugs, the combination of covalent linkages and noncovalent forces between the antibody chains is sufficient to maintain the mAb in an intact form during the analysis. This is due to the mild, nondenaturing conditions of HIC, making it ideal for the analysis of cysteine-linked ADCs. Each peak in Figure 1 corresponds to an intact mAb species with an increasing number of attached drugs molecules (zero to eight bound molecules, D0 to D8). The peaks were identified by comparing the HIC chromatogram to chromatograms found in literature for brentuximab vedotin.<sup>6</sup>

By integrating the peak areas of each peak and its respective drug load, it is possible to calculate the overall DAR (Equation 1).

**Equation 1.**

$$\text{DAR} = \sum_{n=0}^8 \frac{\text{LC peak area} \times n_{\text{drug}}}{\text{Total LC peak area}}$$



**Figure 1.** Analysis of brentuximab vedotin on an Agilent 1290 Infinity II Bio LC. D0 to D8 refers to different DAR species.

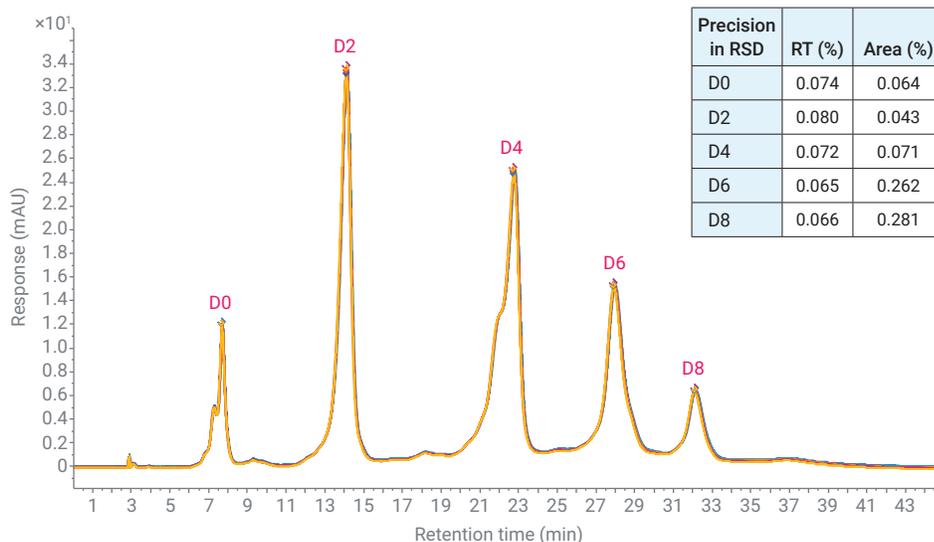
The integration of the five observed peaks and the area percentage calculation revealed a DAR of ~3.7 (see Table 2). This value is consistent with the literature.<sup>8</sup>

The analysis was also evaluated for the precision of retention time (RT) and area (Figure 2). After seven subsequent runs, an excellent RT precision of lower than 0.081% relative standard deviation (RSD) was found. The area precision was also excellent, with RSDs lower than 0.282% (see table in Figure 2).

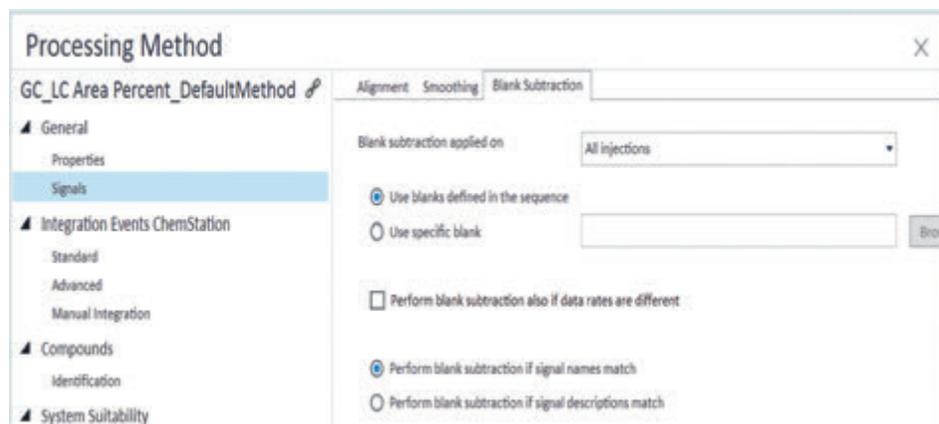
Ammonium sulfate is a very commonly used chaotropic salt in HIC analysis. The concentrations used typically range from 1 to 2 M salt, which is a considerable quantity. If a less pure salt is used in the analysis (which is sometimes even visible in the color of the salt crystals), the baseline of the chromatogram can drift significantly, resulting in potential integration errors. To approach this issue, a software feature called "Blank Subtraction" can be applied to filter out the baseline drift using the blank injection. This feature is found in the processing method of OpenLab 2 (see Figure 3). Figure 4 displays the chromatogram with different baseline behavior before and after the feature was applied.

**Table 2.** DAR species results.

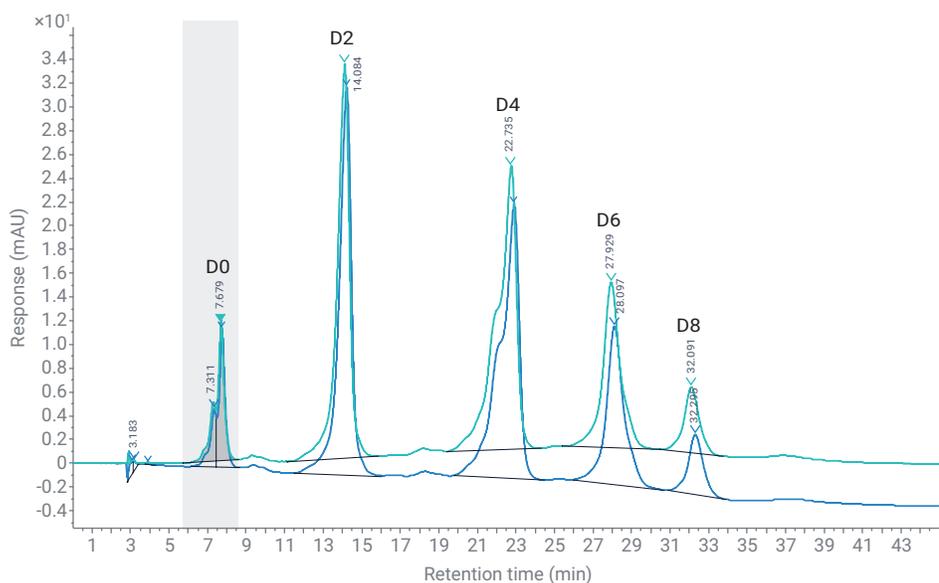
DAR Species	RT (min)	Area	Area%	DAR Calculated
D0	7.68	378.116	7.59	0
D2	14.12	1537.829	30.84	0.6196
D4	22.78	1756.026	35.22	1.415
D6	27.98	951.983	19.13	1.1506
D8	32.15	340.176	6.79	0.5482
			<b>DAR</b>	<b>3.733</b>



**Figure 2.** Separation of brentuximab vedotin on an Agilent 1290 Infinity II Bio LC (overlay of seven subsequent runs).



**Figure 3.** Screenshot of the "Blank Subtraction" feature in the processing method of Agilent OpenLab CDS 2.



**Figure 4.** Comparison of HIC chromatogram before (blue) and after (green) blank subtraction.

## Conclusion

Brentuximab vedotin was analyzed using HIC on the Infinity II 1290 Bio LC. All five expected DAR species were well separated corresponding to the mAb containing zero, two, four, six, and eight small molecule drugs, respectively. The HIC analysis allowed both the characterization of the distribution of drug-linked species as well as the determination of the DAR, calculated to 3.7 drug molecules per antibody. The precision analysis of seven subsequent runs revealed excellent reproducibility for RT and area. The eluents used in HIC contain high concentrations of corrosive salts challenging the LC system. Due to its completely iron-free sample flow path, the 1290 Infinity II Bio LC is optimally suited for the conditions used in biochromatography, avoiding potential corrosive damage to the system.

Blank subtraction as a software feature of OpenLab 2 enables users to employ even less pure ammonium sulfate in their analysis without negatively affecting their results. Just by filtering out the blank runs, the baseline can be corrected to enable smooth integration calculation.

The combination of the biocompatible hardware of the 1290 Infinity II Bio LC with new software features of OpenLab 2 results in the highest confidence in generated data.

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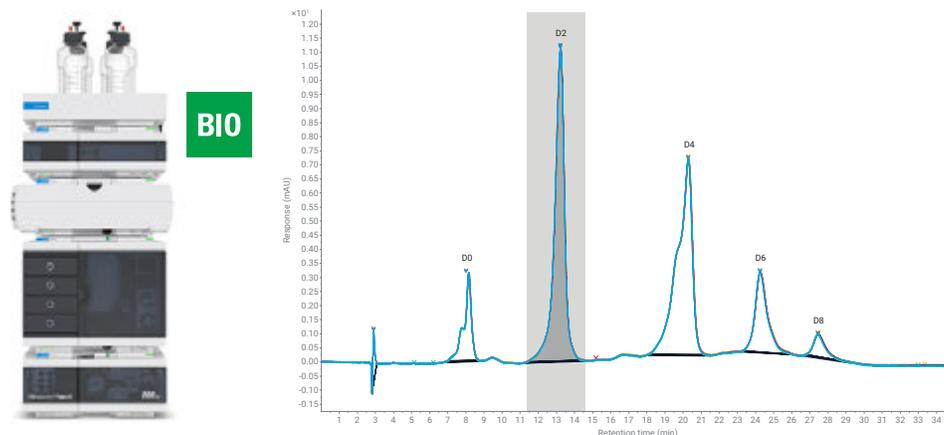
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# Convenient and Reliable Analysis of Antibody Drug Conjugates

Drug-to-antibody determination with ternary gradients on the Agilent 1260 Infinity II Prime Bio LC



## Author

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## Abstract

The addition of organic modifiers like isopropanol in hydrophobic interaction chromatography (HIC) can be an important parameter to decrease the retention of hydrophobic antibody drug conjugates (ADCs) as well as to adjust selectivity. This application note demonstrates the drug-to-antibody (DAR) determination of brentuximab vedotin using a ternary gradient with isopropanol as organic modifier in the third channel. Excellent reproducibility was found for this challenging combination of high-salt-containing buffer and organic solvent, making the Agilent 1260 Infinity II Prime Bio LC the next generation of Agilent high-end liquid chromatography systems for the highest confidence in generated data.

## Introduction

ADCs are monoclonal antibodies (mAbs) to which a cytotoxic small molecule drug is chemically linked.<sup>1</sup> Compared to their corresponding antibodies, the structure is more complex and heterogeneous.

Cysteine-linked ADCs such as brentuximab vedotin are generated after the reduction of the interchain disulfides, resulting in free sulfhydryl groups that can be conjugated to specific maleimide linkers. The number of free sulfhydryl groups limits the number of defined positions for the drug to be conjugated, resulting in a mixture of zero (D0), two (D2), four (D4), six (D6), and eight (D8) drugs per antibody.

Due to the hydrophobicity of the high DAR species in particular, the addition of an organic modifier such as isopropanol is helpful to enable full elution from the HIC column. Typically, in binary gradients, the modifier is added to the mobile phase used for elution (usually a buffer containing little or no salt). The Agilent 1260 Infinity II Bio Flexible Pump, as a quaternary pump, enables the use of a third channel to add the organic modifier solvent. The combination of high-salt-containing buffers with organic mobile phases can be critical due to potential formation of salt crystals when the two solvents mix in the pump.

The 1260 Infinity II Prime Bio LC is the next generation of Agilent high-end liquid chromatography systems, specially designed for conditions used in bio chromatography: The sample flow path is completely free of stainless steel (SST) or iron; all capillaries and fittings throughout the multisampler, multicolumn thermostat, and detectors are made of MP35N, a nickel-cobalt alloy. For this reason, the 1260 Infinity II Prime Bio LC is optimally suited to the

conditions used in bio chromatography, with the high concentrations of corrosive salts typically used in HIC, to avoid potential corrosive damage to the system.

## Experimental

### Equipment

The Agilent 1260 Infinity II Prime Bio LC System comprised the following modules:

- Agilent 1260 Infinity II Bio Flexible Pump (G7131C)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with standard flow biocompatible heat exchanger
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B), equipped with a biocompatible micro flow cell, 3 mm, 2  $\mu$ L

### Software

Agilent OpenLab CDS version 2.5 or later versions

### Column

Agilent AdvanceBio HIC column 3.5  $\mu$ m, 4.6  $\times$  100 mm (part number 685975-908)

### Chemicals

All solvents were LC grade. Isopropanol was purchased from Merck (Darmstadt, Germany). Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22  $\mu$ m membrane point-of-use cartridge (Millipak, Merck-Millipore, Billerica, MA, USA). Sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, and ammonium sulfate were obtained from Sigma-Aldrich (Steinheim, Germany).

## Samples

Brentuximab vedotin (trade name Adcetris by Takeda, Tokyo, Japan) dissolved in 50% water: 50% solvent A (see below) at 100 mg/mL.

**Note:** As Adcetris contains many adjuvants, the concentration mentioned here is not the protein concentration only, but the total concentration of all components of the drug.

### Buffer preparation

A) 1.5 M ammonium sulfate in 50 mM phosphate buffer at pH 7

B) 50 mM phosphate buffer at pH 7

C) Isopropanol

For 2 L of 50 mM phosphate buffer, pH 7, 5.84 g of sodium phosphate monobasic monohydrate and 15.47 g of sodium phosphate dibasic heptahydrate were added to an amber-colored 2 L bottle and filled up to 2 L using ultrapure water. The pH value was checked and adjusted, if necessary, to pH 7 (buffer B). 198.21 g of ammonium sulfate for a total of 1.5 M was added to an empty amber-colored 1 L bottle and filled up to 1 L using the prepared phosphate buffer ( $\rightarrow$  buffer A). The pH value was checked and adjusted, if necessary, to pH 7 (the addition of high amounts of salt can change the pH). Both prepared buffers were filtered using a 0.2  $\mu$ m membrane filter.

## Method

Chromatographic Conditions	
Parameter	Value
Solvent	A) 1.5 M ammonium sulfate in 50 mM phosphate buffer at pH 7 B) 50 mM phosphate buffer at pH 7 C) Isopropanol
Gradient	Gradient: 0 min 55% A, 40% B, 5% C 25 min 0% A, 75% B, 25% C Stop time: 35 min Post time: 10 min
Flow Rate	0.400 mL/min
Temperature	25 °C
Detection	280 nm 10 Hz
Injection	Injection volume: 15 $\mu$ L Sample temperature: 10 °C Needle wash: 3 s in water

**Note:** The high concentrations of salt used in HIC require a robust LC system, and the completely stainless steel (SST)/iron-free flow path of the 1260 Infinity II Prime Bio LC prevents potential corrosion from high-salt-containing buffers. In addition, washing features like seal wash and needle wash help to avoid issues with salt precipitation. However, it is still important to avoid leaving either the LC system or the column in a concentrated salt solution for any length of time.

**Note:** When using concentrated salt solutions as eluents, consider setting corresponding solvent types in the pump method. For example, for solvent A, which features 1.5 M ammonium sulfate, use Ammonium Sulfate 1.5 M rather

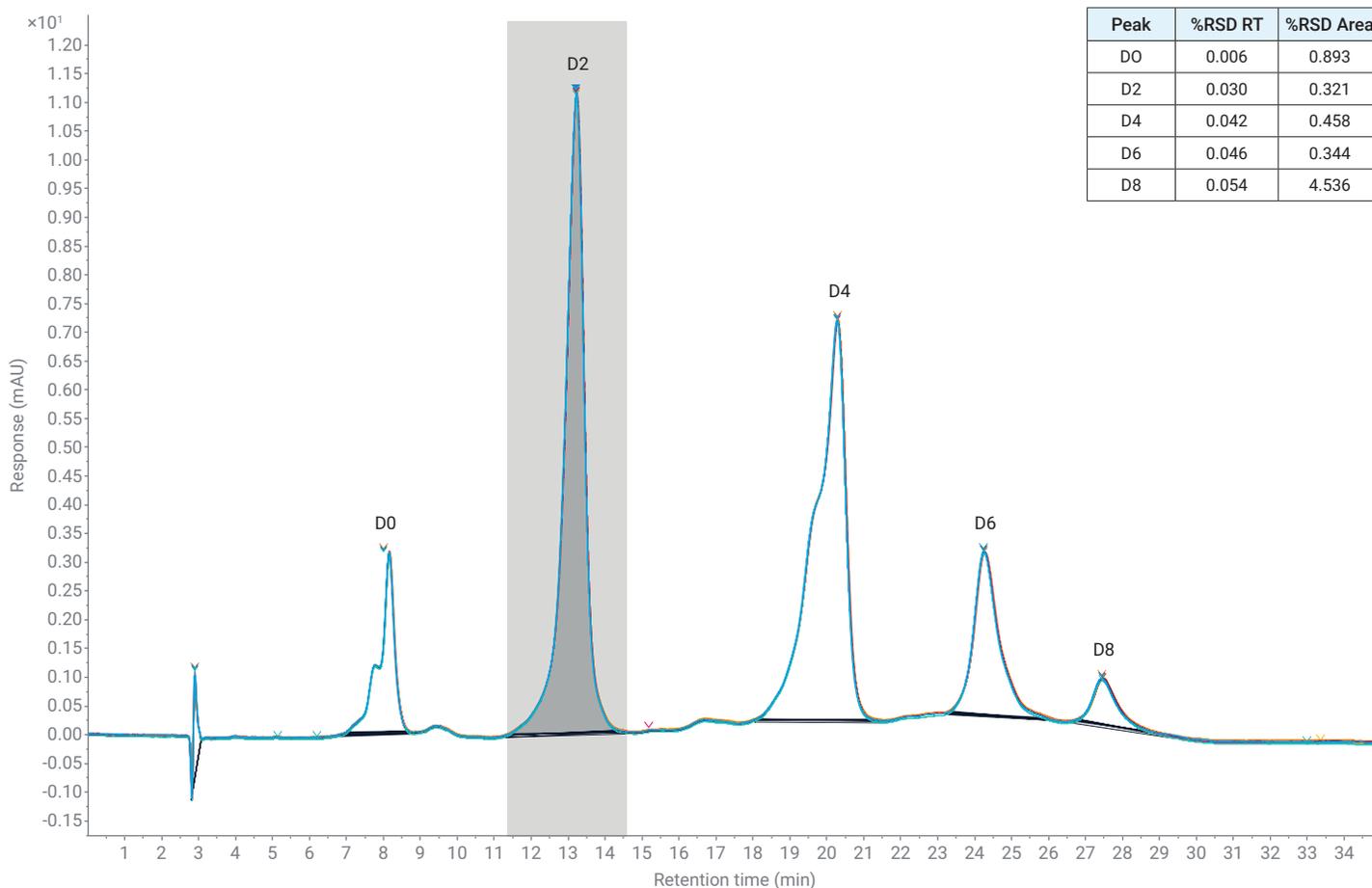
than Generic Aqueous or Water in the solvent selection field in the pump method. High amounts of salts change the compressibility of the solvent, and so using the preconfigured solvent tables enables best pump performance.

## Results and discussion

Figure 1 shows the analysis of brentuximab vedotin with five main peaks that correspond to the mAb containing zero, two, four, six, and eight small molecule drugs, respectively. Each peak in Figure 1 corresponds to an intact mAb species with an increasing number of attached drugs molecules (zero to eight bound molecules, D0 to D8). The peaks were identified by

comparing the HIC chromatogram to chromatograms found in literature for brentuximab vedotin.<sup>2</sup>

The analysis was evaluated for the precision of retention time (RT) and area. With seven subsequent runs, an excellent RT precision of lower than 0.055% relative standard deviation (RSD) was found. This proves the excellence of the quaternary pump to run ternary gradients even with very challenging combinations of high-salt-containing buffers (such as the 1.5 M ammonium sulfate buffer used here) and isopropanol as organic modifier. The area precision was also excellent, with RSDs lower than 0.46%, except for the last peak (see the table in Figure 1).



**Figure 1.** Separation of brentuximab vedotin on the Agilent 1260 Infinity II Prime Bio LC. D0–D8 refers to different DAR species. An overlay of seven subsequent runs is displayed. Blank Subtraction was applied to filter out the baseline drift caused by the ammonium sulfate salt in buffer A using blank injections run in the same sequence.

The HIC analysis allowed both the characterization of the distribution of drug-linked species, as well as the determination of the DAR. By integrating the peak areas of each peak and its respective drug load, it is possible to calculate the overall DAR (Equation 1).

$$\text{DAR} = \sum_{n=0}^8 \frac{\text{LC peak area} \times n_{\text{drug}}}{\text{Total LC peak area}}$$

**Equation 1.**

The integration of the five observed peaks and the area percentage calculation revealed a DAR of ~3.3 (see Table 1).

**Table 1.** DAR species results.

DAR Species	RT (min)	Area	Area%	DAR Calculated
D0	8.00	89.18	8.11	0
D2	13.22	427.04	38.83	0.78
D4	20.29	405.58	36.88	1.48
D6	24.27	140.51	12.78	0.77
D8	27.48	37.38	3.4	0.27
			<b>DAR</b>	<b>3.3</b>

## Conclusion

Brentuximab vedotin was analyzed using HIC in a ternary gradient with isopropanol in a third channel as organic modifier. All five expected ADC species were well separated, corresponding to the mAb containing zero, two, four, six, and eight small molecule drugs, respectively. The HIC analysis allowed both the characterization of the distribution of drug-linked species as well as the determination of the DAR, calculated to 3.3 drug molecules per antibody. The challenging solvent combination was managed outstandingly by the Agilent 1260 Infinity II Prime Bio LC including the Agilent 1260 Infinity II Bio Flexible Pump. The reproducibility of retention times was excellent, with relative standard deviations below 0.055%, allowing binary like performance for highest confidence in generated data.

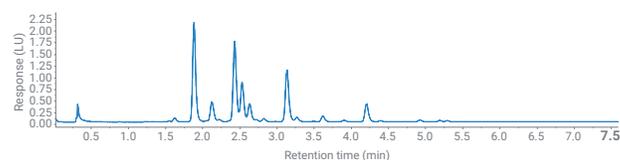
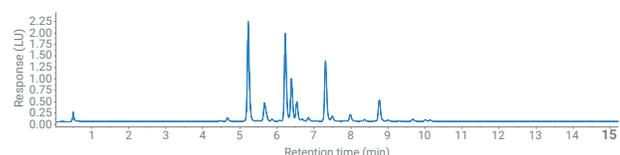
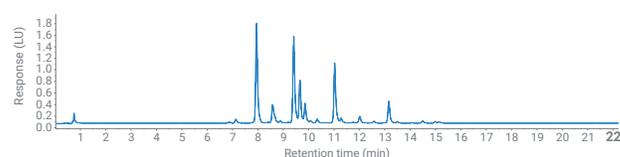
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## Resolution and Speed in the Separation of Glycans



BIO



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### Abstract

This application note demonstrates the reliable separation of 2-AB- and InstantPC-labeled glycans with the Agilent 1290 Infinity II Bio LC System under resolution- and speed-optimized conditions. The reliability is showcased by calculation of performance values such as retention time precision, area precision, and resolution, all of which obtained excellent values. For detection, the Agilent 1260 Infinity II Fluorescence Detector (FLD) with bio-inert flow cell was used. The 1290 Infinity II Bio LC System has low internal volume and the binary pump provides highly precise solvent compositions and flow rates, which enables method transfers from high-resolution to speed-optimized methods with comparable results.

## Introduction

Today, monoclonal antibodies (mAbs) are the most important class of modern biotherapeutic compounds. All therapeutic mAbs belong to the class of immunoglobulins G (IgGs).<sup>1</sup> On their surface, the IgGs have an N-linked glycosylation site on each of their heavy chains, resulting in a mixture of up to 30 glycoforms.<sup>2</sup> Because the glycosylation pattern is important for the therapeutic efficacy of the mAbs, its analysis is a crucial part of the QA/QC process. Moreover, glycosylation is one of the most important critical quality attributes (CQA) for mAbs,

To cope with a larger number of samples, it is necessary to have not only fast sample preparation methods, but also fast analytical methods with sufficient resolution of complex mixtures. Typically, glycans are labeled with fluorescent compounds for their detection with an FLD after chromatographic separation. The labeling methods were accelerated by means of modern labeling compounds such as InstantPC. This label in particular shows a higher fluorescent sensitivity compared to the classical labels, and a good ionization for mass spectrometric detection.<sup>3</sup> The subsequent chromatographic separation was performed on a HILIC column, which is the standard method for the separation of glycans nowadays.<sup>4</sup>

This application note will demonstrate the high-resolution and high-speed separation of a fluorescent-labeled glycan ladder and a fluorescent-labeled human IgG N-glycan library by means of the 1290 Infinity II Bio LC equipped with Agilent AdvanceBio glycan mapping HILIC columns. Typical performance parameters, such as area and retention time RSD, and resolution, will be shown for a 150 and a 100 mm column. As fluorescent labels, the classical 2-AB label and the modern InstantPC label will be compared for the IgG library to show that identical elution patterns can be obtained for the high-resolution and high-speed methods.

## Experimental

### Instrument

- Agilent 1290 Infinity II Bio High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) including integrated Sample Thermostat (#101)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116B) with biocompatible Heat Exchanger
- Agilent 1290 Infinity II Fluorescence Detector (FLD) (G7162B), equipped with bioinert standard FLD flow cell (G5615-60005)

### Columns

1. Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 μm (859700-913)
2. Agilent AdvanceBio Glycan Mapping, 2.1 × 100 mm, 1.8 μm (858700-913)

### Software

Agilent OpenLab version 2.5 and GPC/SEC add-on software V. 1.2

### Samples

- AdvanceBio 2-AB Human IgG N-Glycan Library (part number GKSB-005)
- AdvanceBio InstantPC Human IgG N-Glycan Library (part number GKPC-005)
- AdvanceBio 2-AB Glucose Homopolymer Standard (part number GKSB-503)

### Chemicals

- Acetonitrile, HPLC gradient grade
- Ammonium formate

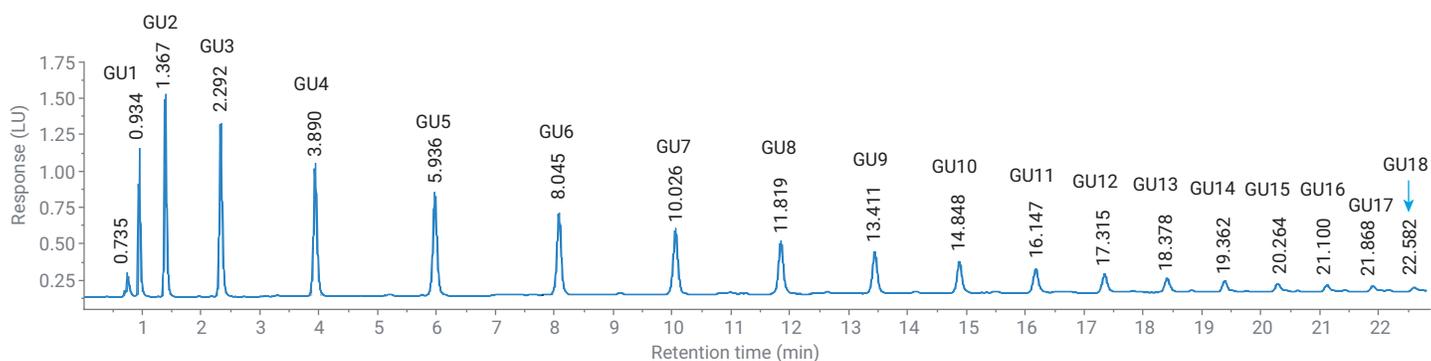
Chemicals were purchased from VWR, Germany. Fresh ultrapure water was obtained from a Milli-Q integral system equipped with LC-Pak polisher and a 0.22 μm membrane point-of-use cartridge (Millipak).

## General method settings

Parameter	Value
<b>Binary Pump</b>	
Solvents	A) water + 100 mM ammonium formate, pH 4.5 B) ACN
<b>MCT</b>	
Column Temperature	60 °C
<b>FLD</b>	
Wavelength (2AB)	Ex. 260 nm, Em. 430 nm
Wavelength (InstantPC)	Ex. 285 nm, Em. 345 nm
Data Rate	18.75 Hz (high-resolution methods)
Data Rate	39.75 Hz (fast-separation method)
<b>Sampler</b>	
Injection	1 µL
Needle Wash	3 s water/ACN 30/70
<b>High-Resolution Method, 150 mm Column</b>	
<b>Binary Pump</b>	
Flow Rate	0.5 mL/in
Gradient	0 min–80% B; 22 min–55% B; 22.25 min–40% B; 24.25 min–40% B; 24.5 min–80% B; stop time: 24.5 min; post time: 7 min
<b>MCT</b>	
	Column 1
<b>High-Resolution Method, 100 mm Column</b>	
<b>Binary Pump</b>	
Flow Rate	0.5 mL/in
Gradient	0 min–80% B; 15 min–55% B; 15.25 min–40% B; 16.75 min–40% B; 17.0 min–80% B; stop time: 17.0 min; post time: 5 min
<b>MCT</b>	
	Column 2
<b>High-Speed Method, 100 mm Column</b>	
<b>Binary Pump</b>	
Flow Rate	0.75 mL/in
Gradient	0 min–75% B; 7.5 min–65% B; 7.75 min–40% B; 8.75 min–40% B; 9.0 min–75% B; stop time: 9.0 min; post time: 3 min
<b>MCT</b>	
	Column 2

## Results and discussion

For the initial development of a high-resolution method on a 150 mm AdvanceBio glycan mapping column, a glucose-based glycan ladder with 2-AB fluorescent label was used. The initial solvent ratio, the end-point solvent ratio, and the run time were determined to have good resolution between residual 2-AB label (0.735 minutes) and glucose unit 1 (GU1, 0.934 minutes) (Figure 1). Glucose homopolymers were separated and detected up to GU18.



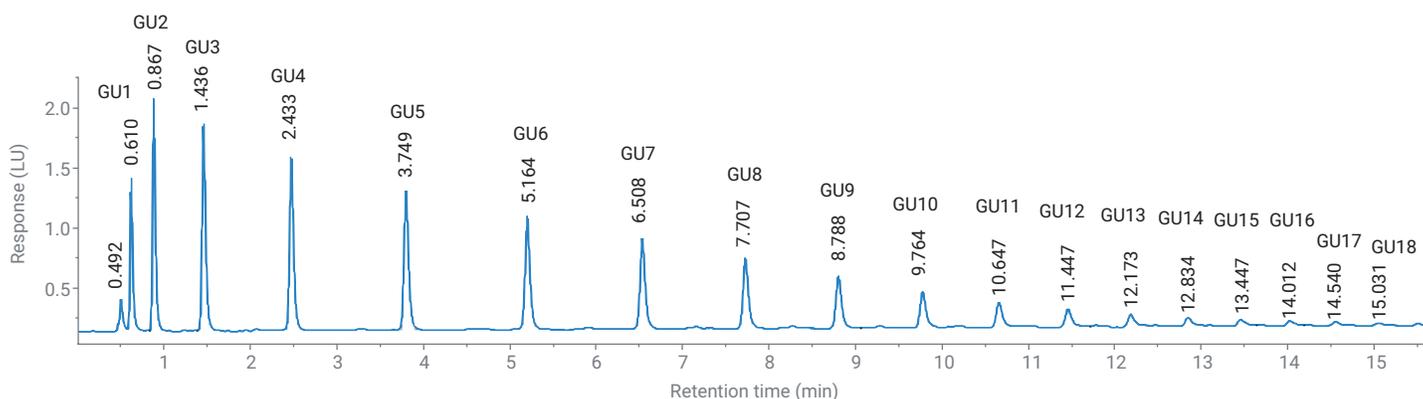
**Figure 1.** High-resolution separation of a glycan ladder on the 150 mm column, adjusted for baseline separation at the beginning for residual 2-AB and GU1 with a generic linear gradient.

The initial resolution of residual 2-AB and GU1 was 4.23, which is clearly baseline separation. The resolution increases up to GU6 at 8.045 minutes. For the later-eluting glucose homopolymers, the resolution declines as the peak width increases. Up to GU5, the area RSD is below 0.65%, up to GU13 between 1.17% and 3.4%. Later-eluting homopolymers show a higher area RSD but correspondingly low peak areas. The retention time RSD is typically below 0.08% for all compounds (Table 1).

The initial high-resolution method was adapted from the 150 mm column to a 100 mm column by decreasing the separation gradient to 15 minutes accordingly. Flushing and regeneration times were adapted to the lower column volume of the 100 mm column. In the obtained separation, the residual 2-AB was still separated from GU1 and the 18 glucose homopolymers were still baseline separated in the gradient run time (Figure 2).

**Table 1.** Performance results of the glycan ladder separation on the 150 mm column.

GU	Area RSD (%)	RT (min)	RT RSD (%)	Resol. USP	Width 50% (min)
1	0.59	0.934	0.06	4.23	0.028
2	0.47	1.367	0.08	8.29	0.034
3	0.64	2.292	0.09	14.03	0.044
4	0.33	3.890	0.08	18.27	0.057
5	0.61	5.936	0.06	18.59	0.069
6	1.23	8.045	0.09	16.89	0.075
7	1.20	10.026	0.08	14.81	0.079
8	1.17	11.819	0.05	12.85	0.082
9	1.50	13.411	0.06	11.17	0.082
10	1.77	14.848	0.05	9.92	0.084
11	2.65	16.147	0.05	8.84	0.085
12	3.40	17.315	0.05	7.92	0.085
13	2.79	18.378	0.05	7.15	0.085
14	6.65	19.362	0.04	6.53	0.089
15	6.44	20.264	0.04	5.99	0.090
16	6.76	21.100	0.03	5.39	0.094
17	6.77	21.868	0.04	4.77	0.093
18	5.57	22.582	0.04	4.52	0.093



**Figure 2.** High-resolution separation of a glycan ladder on the 100 mm column, transferred from 150 mm column by adjusting the gradient to the shorter column. Residual 2-AB and GU1 are still well separated.

The resolution of residual 2-AB and GU1 was 2.16. The resolution also increases up to GU5 eluting at 3.749 minutes. Up to GU4, the area RSD is below 1.00%, up to GU14 between 1.09% and 3.5%. Later-eluting homopolymers show a higher area RSD but correspondingly low peak areas. The retention time RSD is typically below 0.09% for all compounds (Table 2).

By comparison (Tables 1 and 2), the area RSDs and retention time RSDs obtained on the 150 mm and the 100 mm column are in the same order. Resolution, of course, declines when using shorter 100 mm column, but is still high enough for baseline separation of all peaks.

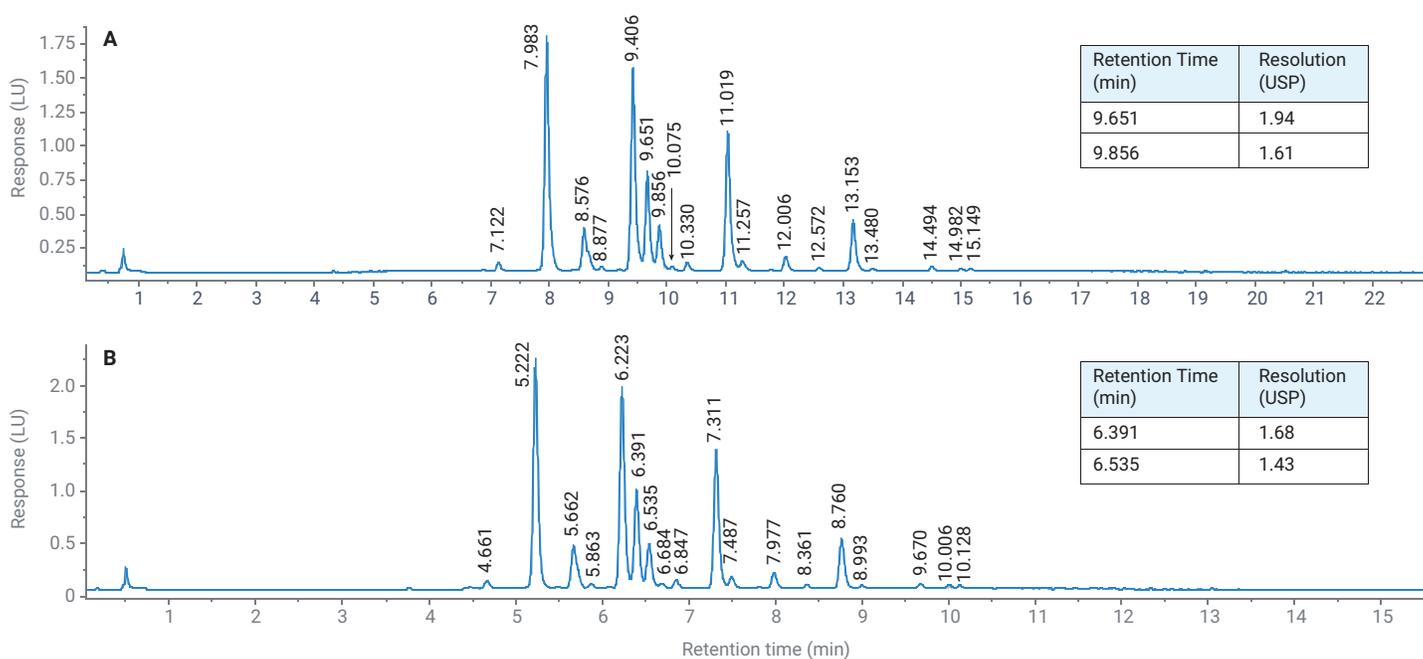
Both high-resolving methods, developed for the 100 mm AdvanceBio glycan mapping column and the 150 mm AdvanceBio glycan mapping column, were applied for the separation of a glycan sample, a fluorescence-labeled N-glycan library of human IgG (Figures 3 and 4). The separations were done with a 2-AB-labeled IgG N-glycan library (Figures 3A and 3B) and an

**Table 2.** Performance results of glycan ladder separation on the 100 mm column.

GU	Area RSD (%)	RT (min)	RT RSD (%)	Resol. USP	Width 50% (min)
1	0.86	0.610	0.13	2.16	0.031
2	0.82	0.867	0.18	4.87	0.032
3	0.95	1.436	0.19	9.23	0.040
4	0.97	2.433	0.09	13.35	0.048
5	1.09	3.749	0.06	15.02	0.054
6	1.13	5.164	0.03	14.41	0.058
7	1.41	6.508	0.08	12.94	0.061
8	1.24	7.707	0.08	11.11	0.063
9	1.57	8.788	0.05	9.59	0.066
10	2.30	9.764	0.04	8.33	0.069
11	3.46	10.647	0.03	7.28	0.071
12	1.82	11.447	0.02	6.32	0.074
13	1.50	12.173	0.03	5.50	0.078
14	3.25	12.834	0.02	4.87	0.079
15	5.92	13.447	0.03	4.25	0.087
16	3.75	14.012	0.03	3.64	0.097
17	5.38	14.540	0.02	3.06	0.105
18	6.17	15.031	0.04	2.82	0.106

InstantPC-labeled IgG N-glycan library (Figures 4A and 4B). The separation of the 2-AB-labeled glycan library shows a comparable elution pattern for the 100 mm and the 150 mm column (Figures 3A and 3B), with the gradient

run time being around one third shorter for the 100 mm column. In both cases, the same low abundance peaks could be observed, and the three peaks that elute in the middle of the pattern were separated with good resolution.



**Figure 3.** High-resolution separation of a 2-AB-labeled IgG N-glycan library on a 150 mm Agilent AdvanceBio glycan mapping column (A) and a 100 mm AdvanceBio glycan mapping column (B).

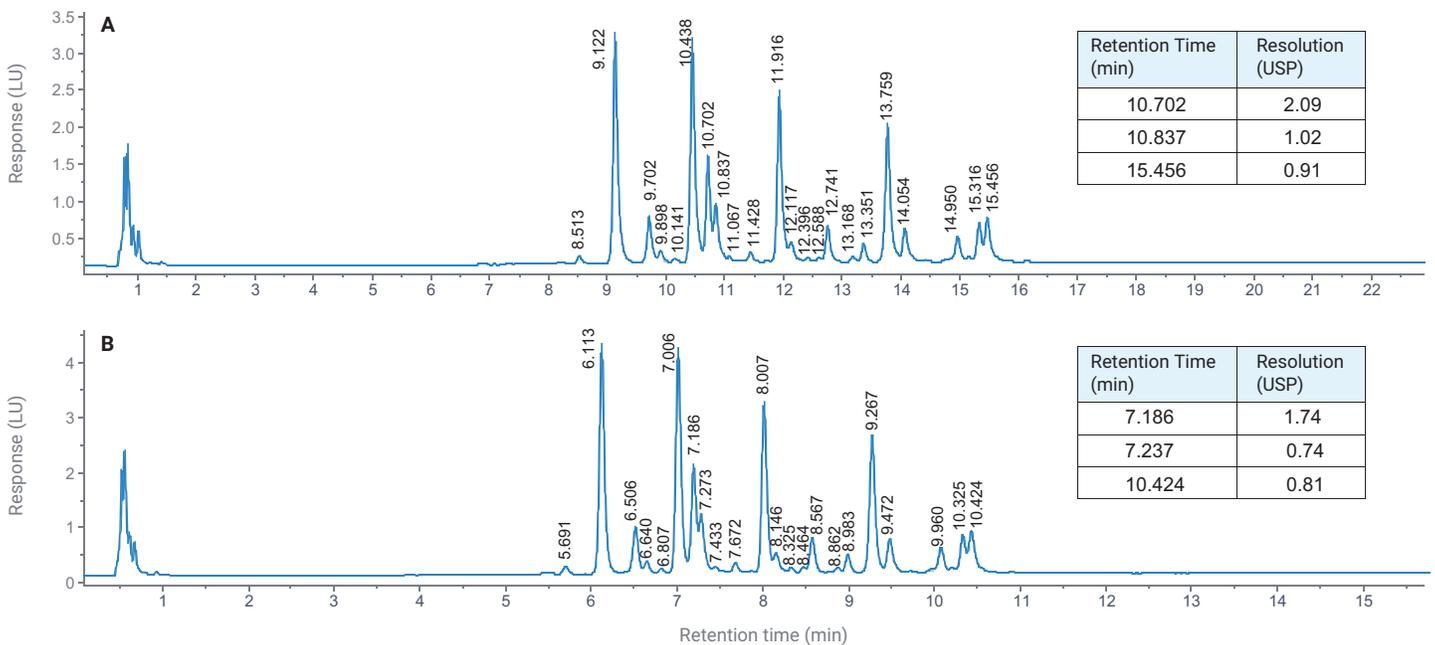
For instance, the peak eluting at 9.651 minutes from the 150 mm column had a resolution value of 1.94 from the peak eluting at 9.408 minutes. On the 100 mm column, this peak elutes at 6.391 minutes with a resolution of 1.68.

The fluorescent label InstantPC is a more recent development with the advantage of higher fluorescent sensitivity and the capability to be ionized for mass spectrometric detection.<sup>3</sup> The elution patterns obtained from the 150 and 100 mm columns were comparable

(Figures 4A and 4B). They provided the same information about the low-level glycans and a comparable resolution for the peaks. In the case of the 100 mm column, the gradient run time was shortened by about one third. In the peak pattern in the middle of the run time, the peak that eluted at 10.702 minutes from the 150 mm column showed a resolution from the peak at 10.438 minutes of 2.09 (Figure 4A). On the 100 mm column, the same peak eluting at 7.186 minutes showed a resolution of 1.74 (Figure 4B).

An identical behavior could be observed for the peaks eluting from the 150 mm and the 100 mm column at 10.837 and 7.237 minutes and for the peaks at 15.456 and 10.424, respectively.

Comparing both applied labels, the InstantPC-labeled separation of the glycan library showed more low-abundance peaks due to its higher fluorescence sensitivity compared to the label 2.



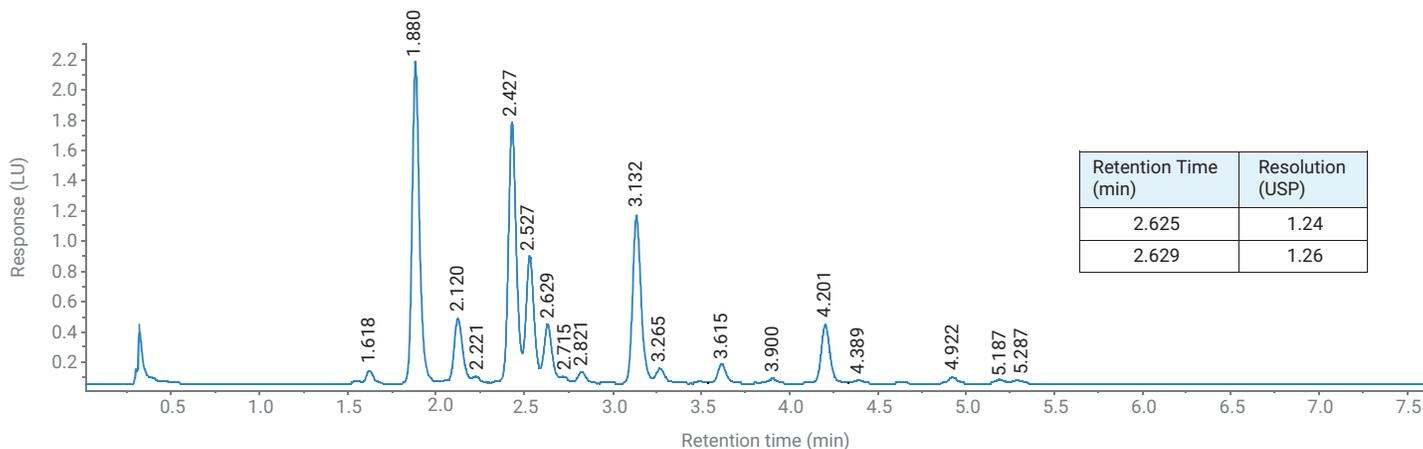
**Figure 4.** High-resolution separation of an InstantPC-labeled IgG N-glycan library on a 150 mm Agilent AdvanceBio glycan mapping column (A) and a 100 mm AdvanceBio glycan mapping column (B).

Since there were improvements in sample preparation, which made it much faster and so accelerated throughput, fast analytical methods were required.<sup>3,4</sup> For the development of a fast glycan separation method, the high-resolution method developed for the 100 mm column was used as the basis. To achieve a shorter run time while retaining the already known elution pattern with sufficient resolution, the flow rate was increased incrementally while the gradient time was decreased accordingly. This led to a method with a gradient run time only half as long as before, at a 50% higher flow rate. With this method, the separation of the

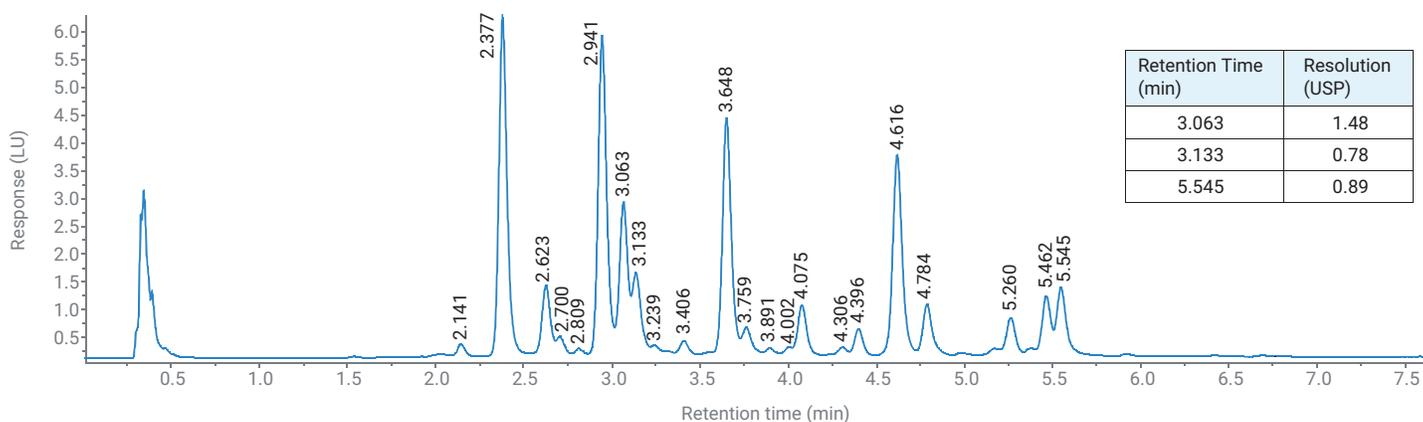
2-AB-labeled and InstantPC-labeled IgG N-glycan library was compared (Figures 5 and 6).

The comparison of the elution pattern obtained for the fast separation of the 2-AB-labeled glycans (Figure 5) with the separation obtained for the high-resolution method (Figure 3B) displays identical elution patterns. Even the resolution obtained for the glycans eluting in the middle of the pattern is sufficient for their visual identification and quantification. The peak that elutes at 2.527 minutes has a sufficient resolution of 1.24 from the previously eluting peak, while resolution was 1.68 for the high-resolution method.

Comparison of the fast separation of InstantPC-labeled glycans (Figure 6) with the high-resolution separation (Figure 4B) also shows comparable elution patterns. The peak eluting at 3.063 minutes had a resolution of 1.48 compared to a resolution of 1.74 obtained for the high-resolution method. Comparing Figures 5 and 6 also shows that the InstantPC-labeled sample provided more information about low abundance glycans as already seen for the high-resolution separations. Due to different chromatographic behavior, the resolution of InstantPC-labeled compounds is compromised in some cases compared to 2-AB-labeled glycans.



**Figure 5.** Separation of the 2-AB-labeled IgG N-glycan library by means of the developed fast separation method achieved on the 100 mm Agilent AdvanceBio glycan mapping column.



**Figure 6.** Separation of the InstantPC-labeled IgG N-glycan library by means of the developed fast separation method achieved on the 100 mm Agilent AdvanceBio glycan mapping column.

## Conclusion

This application note demonstrates the use of the 1290 Infinity II Bio LC for the separation of fluorescent-labeled glycans. The biocompatible UHPLC system enables analyses of biological compounds of high complexity like glycans without the danger of losing lower abundance compounds due to unspecific adsorption on active surfaces in the system.

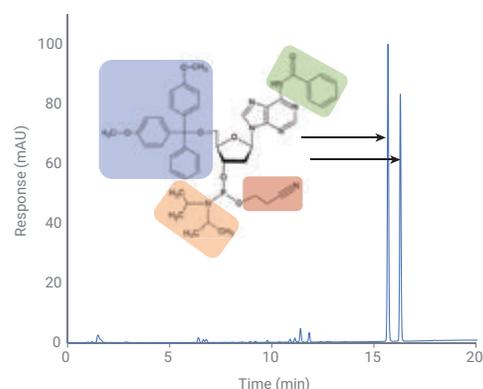
Comparable results can be obtained for method transfers from high-resolution methods to speed-optimized methods due to the low internal delay volume of the 1290 Infinity II Bio LC System, and the highly precise solvent mixing and flow rate provided by the 1290 Infinity II Bio High-Speed Pump. All calculated performance values like retention time precision, area precision, and resolution were obtained with excellent values.

## References

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2. Arnold, J. N. *et al.* Human Immunoglobulin glycosylation and the Lectin Pathway of Complement Activation. *Adv. Exp. Med. Biol.* **2005**, *564*, 27–43.
3. Yan, J.; Jones, A. Streamlined Workflows for N-Glycan Analysis of Biotherapeutics using Agilent AdvanceBio Gly-X InstantPC and 2-AB Express Sample Preparation with FLD/MS. *Agilent Technologies application note*, publication number 5994-1348EN, **2019**.
4. Marosella, J. *et al.* A Novel HILIC Column for High Speed N-linked Glycan Analysis. *Agilent Technologies application note*, publication number 5991-4886EN, **2017**.

# Analyzing Raw Material for Oligonucleotide Synthesis

Flexible and robust method development for the analysis of nucleoside phosphoramidites with the Agilent 1290 Infinity II Bio LC System



## Author

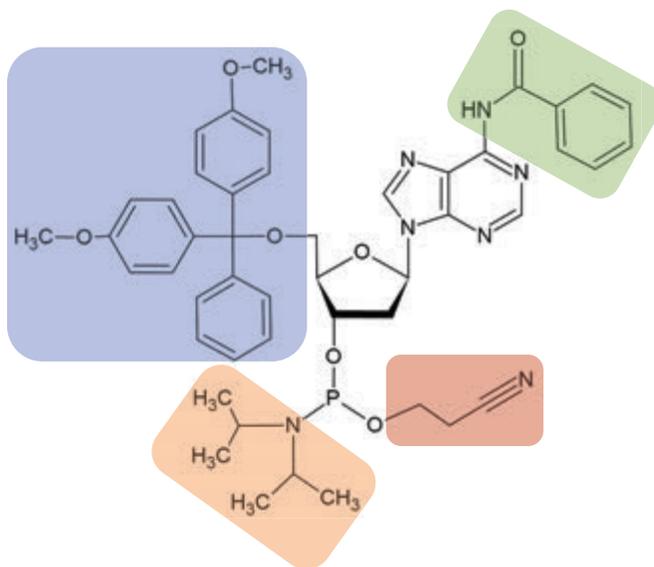
André Feith  
Agilent Technologies, Inc.

## Abstract

As the first step of the biopharmaceutical production process, the raw material of any synthesis needs to be analyzed thoroughly for the best quality products. In the case of oligonucleotides, these raw materials are called nucleoside phosphoramidites and act as building blocks in the subsequent DNA synthesis. In this application note, we developed LC methods based on the biocompatible Agilent 1290 Infinity II Bio LC. By coupling the LC with the Agilent 6545XT AdvanceBio LC/Q-TOF, several impurities could be identified by accurate mass, and method development could be easily carried out by the four-channel Agilent 1290 Infinity II Bio Flexible Pump, which showed excellent retention time and area precision. Based on this, additional method development was carried out to decrease LC run time by 66%, retaining the outstanding performance, and method compatibility experiments were performed, showing seamless method transfer from the conventional Agilent 1290 Infinity II LC. Taken together, these results show that the 1290 Infinity II Bio LC is the perfect choice for the robust and versatile analysis of raw materials for oligonucleotide synthesis.

## Introduction

The chemical synthesis of DNA oligonucleotides has been one of the enabling technologies for modern molecular biology.<sup>1</sup> Combined with the recent approval of several oligonucleotide-based biopharmaceuticals<sup>2</sup>, there is an increased need for reliable and robust analytical methods across the manufacturing and production chain of oligonucleotides. Nucleoside phosphoramidites are considered the gold-standard building blocks in DNA synthesis technology due to the quick and easy removal of the relevant protection groups.<sup>3</sup> Natural nucleosides are rich in reactive sites like hydroxyl (-OH) and amino (-NH<sub>2</sub>) groups. These functional groups are modified in the phosphoramidite building blocks. Figure 1 shows the structural formula of the 5'-DMT-deoxy adenosine 3'-phosphoramidite molecule with four distinct modifications: A dimethoxytrityl (DMT) group (blue) protecting the 5'-hydroxyl group of the desoxyribose, the diisopropylamino (orange) and the 2-cyanoethyl (red) group modifying the phosphoramidite moiety, and a benzoyl (green) group protecting the amino group in the adenine base. After modification, the phosphoramidites of the corresponding DNA nucleoside like deoxyadenosine (dA), deoxyguanosine (dG), deoxycytidine (dC), and deoxythymidine (dT) act as raw material for the subsequent automated oligonucleotide synthesis with multiple synthesis cycles consisting of distinct deprotection, coupling, oxidation, and capping steps.<sup>1</sup> However, the purity and impurities of the raw material need to be closely monitored and identified to minimize sequence impurities and improve coupling efficiencies.



**Figure 1.** Schematic overview of the modified groups in the 5'-DMT-deoxy adenosine 3'-phosphoramidite molecule. Blue: dimethoxytrityl (DMT) group. Green: benzoyl group. Orange: diisopropylamino group. Red: 2-cyanoethyl group.

## 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 LC System:

- Agilent 1290 Infinity II Bio Flexible Pump (G7131A)
- 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  $\mu$ L, RFID.
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6545XT)

The Agilent 1290 Infinity II LC System comprised the following modules:

#### Agilent 1290 Infinity II LC:

- Agilent 1290 Infinity II Flexible Pump (G7104A)
- Agilent 1290 Infinity II Multisampler (G7167A) with Sample Thermostat (option 101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) equipped with a Standard Flow Quick Connect Heat Exchanger (G7116-60015) and two Agilent Thermal Equilibration Devices (G7116-60013)
- Agilent 1290 Infinity II Variable Wavelength Detector (VWD) (G7114B), equipped with a Micro Flow Cell VWD, 3 mm, 2  $\mu$ L, RFID.

#### Software

- Agilent MassHunter workstation data acquisition (B.09.00 or later)
- Agilent MassHunter qualitative analysis (10.0 or later)

## Column

Agilent ZORBAX RRHD Eclipse Plus C18, 95 Å, 2.1 × 100 mm, 1.8 µm (part number 959758-902)

## Chemicals

InfinityLab Ultrapure LC/MS acetonitrile (part number 5191-4496) and the InfinityLab Ultrapure LC/MS water (part number 5191-4498) were used. Ammonium acetate and acetic acid were obtained from VWR (Darmstadt, Germany).

## Sample preparation

Authentic 5'-O-DMT 2' deoxyadenosine phosphoramidite (dA) raw material for the synthesis of oligonucleotides was provided by the Agilent Nucleic Acid Solutions Division. The injection concentration was 1 mg/mL phosphoramidite in acetonitrile.

**Table 3.** Source and MS parameters for the impurity analysis of 5'-DMT-deoxy adenosine 3'-phosphoramidite raw material with the Agilent 1290 Infinity II Bio LC.

Parameter	Value
Instrument	Agilent 6545XT AdvanceBio LC/Q-TOF
Gas Temperature	320 °C
Drying Gas Flow	8 L/min
Nebulizer	35 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
VCap	3,500 V
Nozzle Voltage	1,000 V
Fragmentor	140 V
Skimmer	65 V
Oct 1 RF Vpp	750 V
Acquisition Mode	Positive, extended ( <i>m/z</i> 10,000) mass range
Mass Range	<i>m/z</i> 25 to 10,000
Acquisition Rate	1 spectrum/sec
Reference Mass	<i>m/z</i> 121.050873, <i>m/z</i> 922.0098

**Table 1.** Method A for the impurity analysis of 5'-DMT-deoxy adenosine 3'-phosphoramidite raw material with the Agilent 1290 Infinity II Bio LC.

Parameter	Value																																
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 95 Å, 2.1 × 100 mm, 1.8 µm																																
Solvent	A) Acetonitrile B) Water C) 500 mM ammonium acetate, pH 5.5																																
Gradient	<table border="1"><thead><tr><th>Time (min)</th><th>A (%)</th><th>B (%)</th><th>C (%)</th></tr></thead><tbody><tr><td>0.00</td><td>50</td><td>48</td><td>2</td></tr><tr><td>1.00</td><td>50</td><td>48</td><td>2</td></tr><tr><td>15.00</td><td>90</td><td>8</td><td>2</td></tr><tr><td>15.01</td><td>90</td><td>8</td><td>2</td></tr><tr><td>18.00</td><td>90</td><td>8</td><td>2</td></tr><tr><td>18.01</td><td>50</td><td>48</td><td>2</td></tr><tr><td>25.00</td><td>50</td><td>48</td><td>2</td></tr></tbody></table>	Time (min)	A (%)	B (%)	C (%)	0.00	50	48	2	1.00	50	48	2	15.00	90	8	2	15.01	90	8	2	18.00	90	8	2	18.01	50	48	2	25.00	50	48	2
Time (min)	A (%)	B (%)	C (%)																														
0.00	50	48	2																														
1.00	50	48	2																														
15.00	90	8	2																														
15.01	90	8	2																														
18.00	90	8	2																														
18.01	50	48	2																														
25.00	50	48	2																														
Flow Rate	0.200 mL/min																																
Temperature	20 °C with thermal equilibration devices installed																																
UV Detection	VWD: 236 nm, 10 Hz/MS: see Table 2																																
Injection	Injection volume: 2 µL Sample temperature: 4 °C Wash: 3 s with 90% acetonitrile/10% water (flush port)																																

**Table 2.** Method B with optimized run time for the impurity analysis of 5'-DMT-deoxy adenosine 3'-phosphoramidite raw material with the Agilent 1290 Infinity II Bio LC.

Parameter	Value																																
Column	Agilent ZORBAX RRHD Eclipse Plus C18, 95 Å, 2.1 × 100 mm, 1.8 µm																																
Solvent	A) Acetonitrile B) Water C) 500 mM ammonium acetate, pH 5.5																																
Gradient	<table border="1"><thead><tr><th>Time (min)</th><th>A (%)</th><th>B (%)</th><th>C (%)</th></tr></thead><tbody><tr><td>0.00</td><td>50</td><td>48</td><td>2</td></tr><tr><td>0.33</td><td>50</td><td>48</td><td>2</td></tr><tr><td>5.00</td><td>90</td><td>8</td><td>2</td></tr><tr><td>5.01</td><td>90</td><td>8</td><td>2</td></tr><tr><td>6.00</td><td>90</td><td>8</td><td>2</td></tr><tr><td>6.01</td><td>50</td><td>48</td><td>2</td></tr><tr><td>8.33</td><td>50</td><td>48</td><td>2</td></tr></tbody></table>	Time (min)	A (%)	B (%)	C (%)	0.00	50	48	2	0.33	50	48	2	5.00	90	8	2	5.01	90	8	2	6.00	90	8	2	6.01	50	48	2	8.33	50	48	2
Time (min)	A (%)	B (%)	C (%)																														
0.00	50	48	2																														
0.33	50	48	2																														
5.00	90	8	2																														
5.01	90	8	2																														
6.00	90	8	2																														
6.01	50	48	2																														
8.33	50	48	2																														
Flow Rate	0.600 mL/min																																
Column Temperature	20 °C with thermal equilibration devices installed																																
UV Detection	VWD: 236 nm, 10 Hz																																
Injection	Injection volume: 2 µL Sample temperature: 4 °C Wash: 3 s with 90% acetonitrile / 10% water (Flush Port)																																

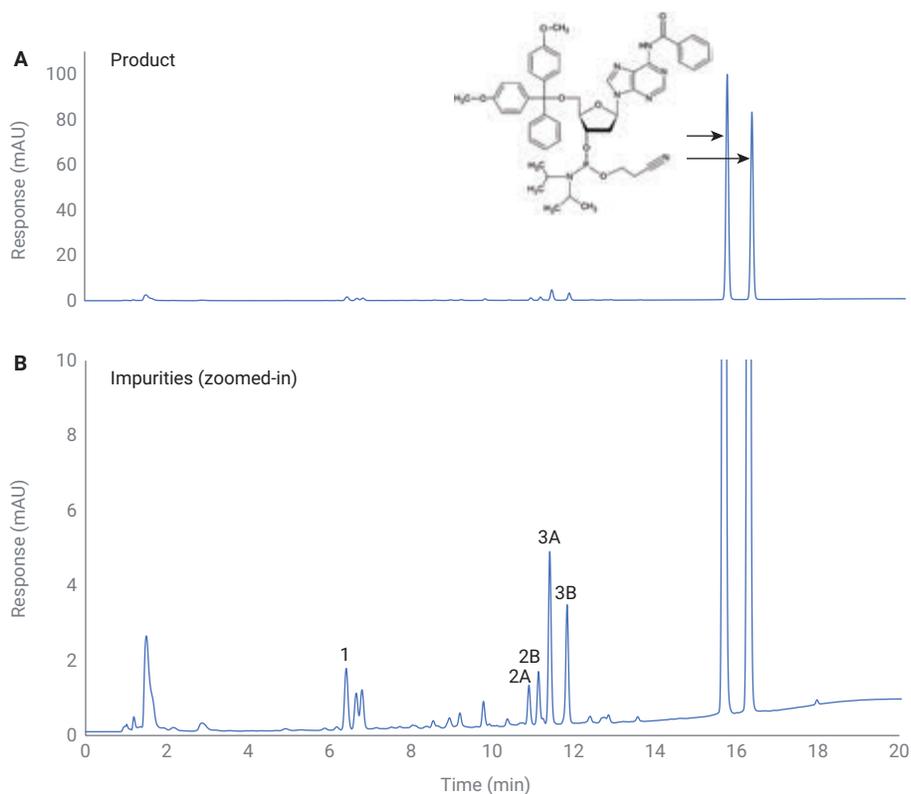
## Results and discussion

HPLC method development and method transfer into different business units, like early process development and quality control, can be tedious and cumbersome. However, owing to the versatility that the 1290 Infinity II Bio Flexible Pump lends to the 1290 Infinity II Bio LC System, method development can be convenient and robust. To showcase this, an impurity analysis LC method for the 5'-DMT-deoxy adenosine 3'-phosphoramidite raw material was developed by employing a ternary gradient on the four-channel Infinity II Bio Flexible Pump. Due to the corrosion-resistant flow path of the 1290 Infinity II Bio LC, highly concentrated buffers like 500 mM ammonium acetate (pH 5.5) can be used routinely for method development, which enables the fast screening of different buffer amounts and types with on-the-fly gradient mixing (Table 1, Method A). The product and three impurities (Table 4) could be identified with accurate mass by coupling the 1290 Infinity II Bio LC with the 6545XT AdvanceBio LC/Q-TOF sequentially, which allowed simultaneous UV and MS analysis of the compounds. Due to an epimerization at the chiral center of the phosphorus atom of the phosphoramidite, two peaks (A and B) are visible for the product, impurity 2 and impurity 3.

Figure 2 shows chromatograms of the raw material separation detected with the VWD equipped with the Bio Micro Flow Cell. The lower chromatogram depicts a zoomed-in view of the impurities with good resolution between the peaks of interest. The analysis of the 3'-DMT-deoxy adenosine 5'-phosphoramidite (impurity 2A/2B) is especially critical since the so-called "reverse amidite" can lead to errors in the subsequent DNA synthesis.

**Table 4.** Product and impurities identified in the 5'-DMT-deoxy adenosine 3'-phosphoramidite raw material.

Name	Species	Retention Time (min)
5'-DMT-Deoxy Adenosine 3'-Phosphoramidite	Product A	15.69
	Product B	16.29
5'-DMT-Deoxy Adenosine	Impurity 1	6.40
3'-DMT-Deoxy Adenosine 5'-Phosphoramidite (Reverse Amidite)	Impurity 2A	10.89
	Impurity 2B	11.13
5'-DMT-Deoxy Adenosine Phosphoramidate	Impurity 3A	11.41
	Impurity 3B	11.83



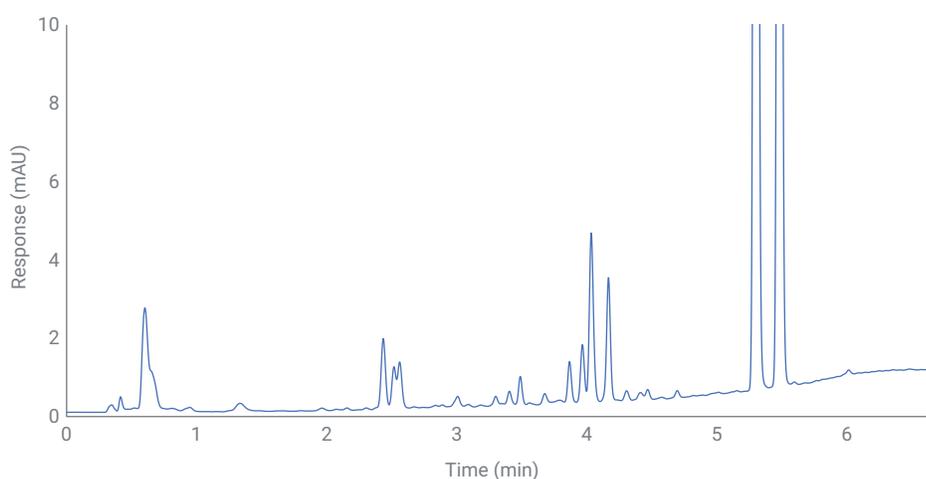
**Figure 2.** A: Chromatogram of the LC separation (method A) of the raw material with the Agilent 1290 Infinity II Bio LC, highlighting the product. B: Zoomed-in view of the same chromatogram for better visibility of the impurities.

Ten consecutive injections were analyzed to further investigate the 1290 Infinity II Bio LC performance, and retention time and area precision values were calculated (Table 5). Excellent retention time RSD values between 0.015% and 0.050% for the product and impurities were determined, showcasing the outstanding performance of the 1290 Infinity II Bio Flexible Pump when deploying a challenging ternary gradient separation. Additionally, the 1290 Infinity II Bio LC's performance in terms of area precision was also excellent, with an average relative standard deviation of 0.269%. To further evaluate the method compatibility of the 1290 Infinity II Bio LC with the 1290 Infinity II LC, the previous experiments were also run on the latter. Table 5 shows that the absolute retention times on both LC systems only deviated with an average of 0.686%, which shows excellent method transferability from the 1290 Infinity II LC.

Time is a critical resource in modern laboratories, especially for routine analysis in the quality control of biopharmaceuticals and their raw materials. Hence, further method development was employed to reduce the run time of the LC analysis. Because of the high-pressure rating of up to 1,300 bar for the 1290 Infinity II Bio LC System, high flow rates and small particles can be used to optimize performance and run time. By increasing the flow rate to 0.6 mL/min and keeping the gradient volume constant, the LC method was shortened by 66% to only 8.33 minutes (Table 2, Method B). Even though the run time was decreased, the excellent performance remained with high retention time and area precision (Figure 3 and Table 6), rendering the 1290 Infinity II Bio LC the perfect fit for versatile and robust analytical method development across the production chain of oligonucleotides.

**Table 5.** Retention time and area precision values for the compounds analyzed with method A and absolute retention time deviations compared between the Agilent 1290 Infinity II Bio LC and Agilent 1290 Infinity II LC.

Species	Retention Time RSD (%)	Area RSD (%)	Retention Time Deviation 1290 Bio LC Versus 1290 LC (%)
Product A	0.017	0.113	0.07
Product B	0.015	0.118	0.19
Impurity 1	0.050	0.211	1.53
Impurity 2A	0.026	0.314	0.85
Impurity 2B	0.020	0.539	0.76
Impurity 3A	0.022	0.209	0.73
Impurity 3B	0.021	0.378	0.67



**Figure 3.** Zoomed-in view of the LC separation with shorter run time (method B) of the raw material with the Agilent 1290 Infinity II Bio LC.

**Table 6.** Retention time and area precision values for the compounds analyzed with method B.

Species	Retention Time (min)	Retention Time RSD (%)	Area RSD (%)
Product A	5.30	0.042	0.180
Product B	5.48	0.40	0.185
Impurity 1	2.43	0.115	0.214
Impurity 2A	3.86	0.079	0.557
Impurity 2B	3.96	0.066	0.986
Impurity 3A	4.03	0.065	0.355
Impurity 3B	4.16	0.059	0.301

## Conclusion

Even though the DNA synthesis technology based on phosphoramidites has been around since the 1980s, streamlining related analytical challenges is still an important task. In this application note, we showed the ability of the Agilent 1290 Infinity II Bio LC with Flexible Pump to perform impurity analysis on the authentic raw material of the 5'-DMT-deoxy adenosine 3'-phosphoramidite building block with the highest confidence in process development and quality control environments. Thanks to the versatility and high performance of the Agilent 1290 Infinity II Bio Flexible Pump combined with the entire 1290 Infinity II Bio LC System's biocompatible flow path, excellent retention time and area precision were achieved, and seamless method compatibility was observed. That is why the 1290 Infinity II Bio LC System is a future-proof choice for challenging analyses of biomolecules.

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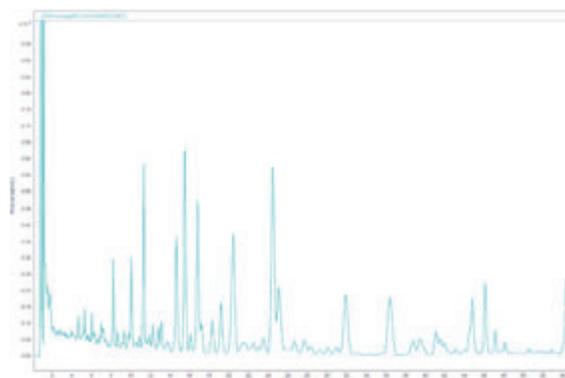
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# Performance Comparison to Determine LC Method Compatibility

Comparative study between Agilent 1260 Infinity II Prime LC System and Agilent 1260 Infinity II Prime Bio LC System

**BIO**

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## Abstract

The new Agilent 1260 Infinity II Prime Bio LC System is equipped with a quaternary pump with an 800-bar pressure rating, facilitating a huge number of applications. Suitable applications are the small-molecule-dominated reversed-phase LC application space, as well as the biomolecular space with native protein and nucleic acid applications. The performance of this completely iron-free, robust, and versatile liquid chromatography system is compared to its stainless-steel-based equivalent, the Agilent 1260 Infinity II Prime LC System.

Isocratic, fast gradient, and shallow gradient applications were used to address performance comparison in terms of pump mixing precision and also method compatibility. All applications demonstrated very good reproducibility on both systems. Excellent method compatibility was found for isocratic and fast gradient applications, whereas the shallow gradient method needs some adjustment due to the high impact of small mobile phase composition changes on retention times (RT) of the sample constituents.

## Introduction

The number of applications using biomolecules like proteins and nucleic acids as samples in (U)HPLC is steadily increasing. This raises concerns about the usefulness and robustness of the LC hardware, especially when it comes to high-salt buffers as mobile phases and harsh cleaning-in-place solvents like hydrochloric acid, sodium hydroxide, and urea. There is a clear need for robust and reliable instrumentation that can withstand these run and cleaning conditions to give highest performance for both bio-applications and standard reversed-phase applications.

The 1260 Infinity II Prime Bio LC System is one of a family of universal systems consisting of an 800-bar quaternary pump as the core solvent delivery module. Its counterpart in the standard reversed-phase application space is the 1260 Infinity II Prime LC System. Both systems share the same performance specifications.

Method compatibility is a strong argument for customers when buying new LC instrumentation, possibly as a replacement for an older Agilent LC system. Since the performance specifications for both 1260 Infinity II Prime systems are the same, method compatibility is generally possible.

This technical overview compares the pump performance of the iron-free and biocompatible 1260 Infinity II Prime Bio LC System and the stainless steel (SST)-based 1260 Infinity II Prime LC System in a number of different applications (i.e., in isocratic, fast gradient, and shallow-gradient mode).

## Experimental

### Equipment

The Agilent 1260 Infinity II Prime Bio LC System comprised the following modules:

- Agilent 1260 Infinity II Bio Flexible Pump (G7131C)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with the standard flow biocompatible heat exchanger
- Agilent 1290 Infinity II DAD (G7117B), equipped with a biocompatible InfinityLab Max-Light Cartridge Cell, 10 mm

The Agilent 1260 Infinity II Prime LC System contains the following modules:

- Agilent 1260 Infinity II Flexible Pump (G7104C)
- Agilent 1290 Infinity II Multisampler (G7167B) with Sample Thermostat (option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity II DAD (G7117B)

### Software

Agilent OpenLab CDS version 2.5 or later versions.

### Columns

- **Isocratic method:** Agilent ZORBAX Eclipse Plus C18, 4.6 × 150 mm, 5 μm (part number 959993-902)
- **Fast gradient method:** Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 50 mm, 2.7 μm (part number 699775-902)
- **Shallow gradient method:** Agilent InfinityLab Poroshell 120 SB-C18, 2.1 × 150 mm, 2.7 μm (part number 683775-902)

### Chemicals

All solvents were LC grade. 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). Acetonitrile, Lichrosolv and methanol, Lichrosolv were obtained from VWR International, Darmstadt, Germany.

### Samples

- Agilent Isocratic sample (part number 01080-68704)
- Agilent RRLC mix (part number 5188-6529)
- Ganoderma Lucidum Fruiting Body Dry Extract (USP Reference Standard, Catalog No. 1288372) 15.5 mg Ganoderma Lucidum Fruiting Body Dry Extract was dissolved in 7.75 mL MeOH, vortexed, and sonicated. An approximately fourfold concentration step in a vacuum concentrator, followed by centrifugation and filtration through a 0.22 μm syringe filter, led to the final sample solution.

## Methods

### Isocratic method

**Table 1.** Isocratic method conditions.

Parameter	Value
Solvent	A) Water B) Acetonitrile (ACN) C) ACN D) Water
Gradient	Isocratic, 65% B (channel A/B) or 65% C (channel C/D) Stop time: 20 minutes Post-time: off
Pump Settings	Minimum stroke: automatic Compressibility: $60 \times 10^{-6}$ /bar Primary channel: automatic
Flow Rate	1.000 mL/min
Temperature	40 °C
Detection	254 nm 10 Hz
Injection	Injection volume: 5 µL Sample temperature: 10 °C Needle wash: 3 s in ACN/water (50/50)

### Fast gradient method

**Table 2.** Fast gradient method conditions.

Parameter	Value
Solvent	A) water B) ACN
Gradient	0 min 20% B 5 min 90% B Stop time: 6 min Post-time: 3 min
Pump Settings	Minimum stroke: automatic Compressibility: $55 \times 10^{-6}$ /bar Primary channel: automatic
Flow Rate	1.000 mL/min
Temperature	40 °C
Detection	245 nm 40 Hz
Injection	Injection volume: 5 µL Sample temperature: 10 °C Needle wash: 3 s in ACN/water (50/50)

### Shallow gradient method

**Table 3.** Shallow gradient method conditions.

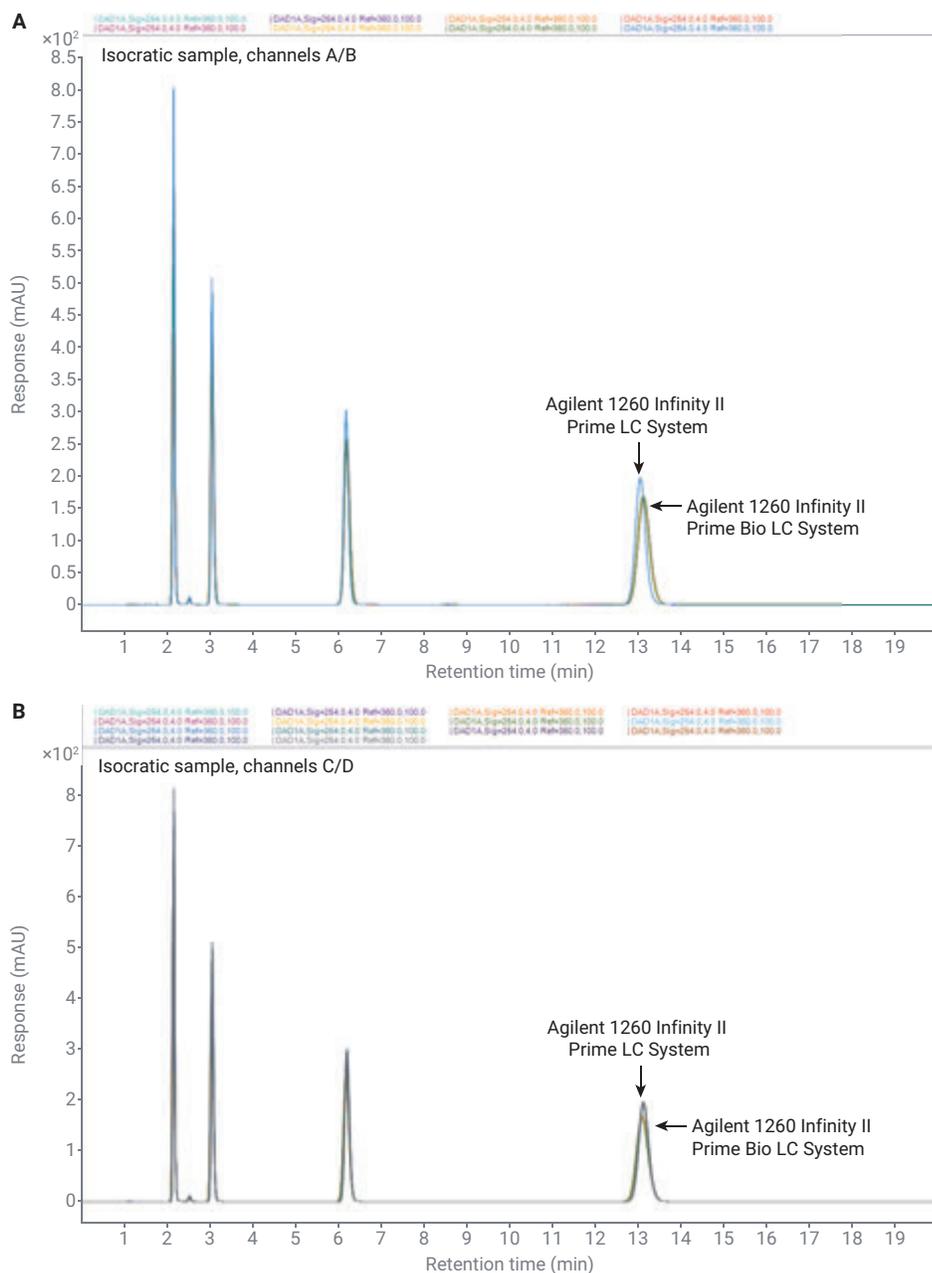
Parameter	Value
Solvent	A) 0.075% phosphoric acid in water B) ACN
Gradient	0 min 20% B 3 min 26.5% B 34 min 26.5% B 52 min 38.5% B 54 min 100% B Stop time: 55 min Post-time: 15 min
Pump Settings	Minimum stroke: automatic Compressibility: $50 \times 10^{-6}$ /bar Primary channel: automatic
Flow Rate	0.400 mL/min
Temperature	30 °C
Detection	257 nm 20 Hz
Injection	Injection volume: 5 µL Sample temperature: 10 °C Needle wash: 3 s in ACN/water (50/50)

## Results and discussion

### Isocratic method

With this application, the capability of the pumps to mix the mobile phase for separating the components of the isocratic sample in an accurate, stable, and precise manner was addressed. Repeatability and method compatibility was tested by running 10 injections of isocratic sample, where one sequence was executed with solvent channels A/B and the second sequence with C/D, as solvent lines for mixing the mobile phase. The last seven consecutive chromatograms from each sequence were used for evaluation (see Figure 1).

The same method was run on both systems. The overlay of corresponding chromatograms demonstrates excellent repeatability in terms of peak retention times on both channel combinations with both systems. Small differences in peak shape are related to tiny variances between the instruments.



**Figure 1.** Overlay of seven subsequent chromatographic runs of the isocratic sample on the Agilent 1260 Infinity II Prime LC System and the Agilent 1260 Infinity II Prime Bio LC System. (A) data for channels A/B; (B) data for channels C/D.

The solvent composition precision specification for both systems is defined as <0.15% RSD, or 0.02 minutes SD, whatever is greater. Table 4 shows the composition precision RSD values for the isocratic sample at the given retention times of its constituents. High pump performance could be confirmed, no matter which solvent channel combination was used. The method compatibility is outstanding, with RT deviations far below the  $\pm 1\%$  range, thereby demonstrating a high degree of performance match between the two instruments.

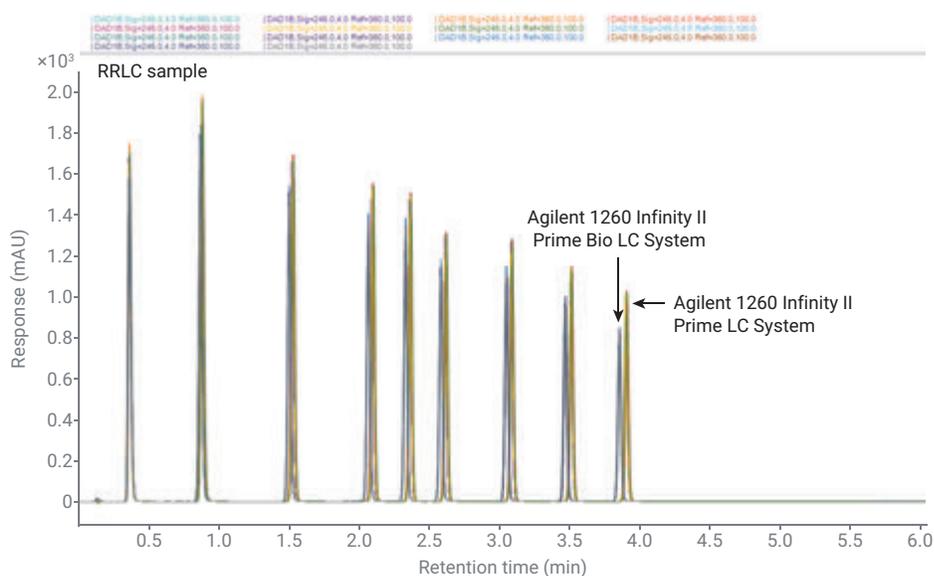
### Fast gradient method

UHPLC methods generally feature faster and steeper solvent composition gradients using small particle columns running at higher backpressures. In this case, a 5-minute gradient starting from 20% B ranging up to 90% B and a run time of 6 minutes was applied. Overlaying seven UV traces generated by both systems revealed very good reproducibility of pump mixing precision (Figure 2, Table 5).

Retention time differences between the two systems become visible, especially at the later eluting peaks. However, the relative retention time deviation (%RT) of eluting sample components is well below 2% between the two instruments, demonstrating valuable method compatibility.

**Table 4.** Method compatibility and retention time (RT) precision evaluation for both solvent channels. Average RT values out of seven sample injections from both LC systems were used. For method compatibility, the values for the Agilent 1260 Infinity II Prime LC System were used as a reference.

Solvent Channels	Retention Time 1260 Agilent Infinity II Prime LC System (min)	RT % RSD	Retention Time 1260 Agilent Infinity II Prime Bio LC System (min)	RT % RSD	RT Deviation (min)	RT Deviation (%)
A/B	2.1533	0.0408	2.1487	0.0164	0.0046	-0.2134
A/B	3.0430	0.0361	3.0431	0.0771	0.0000	0.0012
A/B	6.1627	0.0226	6.1780	0.0417	-0.0153	0.2478
A/B	13.0088	0.0236	13.0808	0.0235	-0.0720	0.5537
C/D	2.1579	0.0536	2.1494	0.0385	0.0085	-0.3920
C/D	3.0534	0.0632	3.0435	0.0523	0.0099	-0.3244
C/D	6.1957	0.0349	6.1791	0.0380	0.0165	-0.2668
C/D	13.1101	0.0190	13.0814	0.0232	0.0287	-0.2191



**Figure 2.** Overlay of seven subsequent chromatographic runs of the RRLC sample on the Agilent 1260 Infinity II Prime LC System and the Agilent 1260 Infinity II Prime Bio LC System.

**Table 5.** Method compatibility and retention time (RT) precision evaluation with RRLC sample. Average RT values out of seven sample injections from both LC systems were used. For method compatibility estimation, the values for the Agilent 1260 Infinity II Prime LC System were used as a reference.

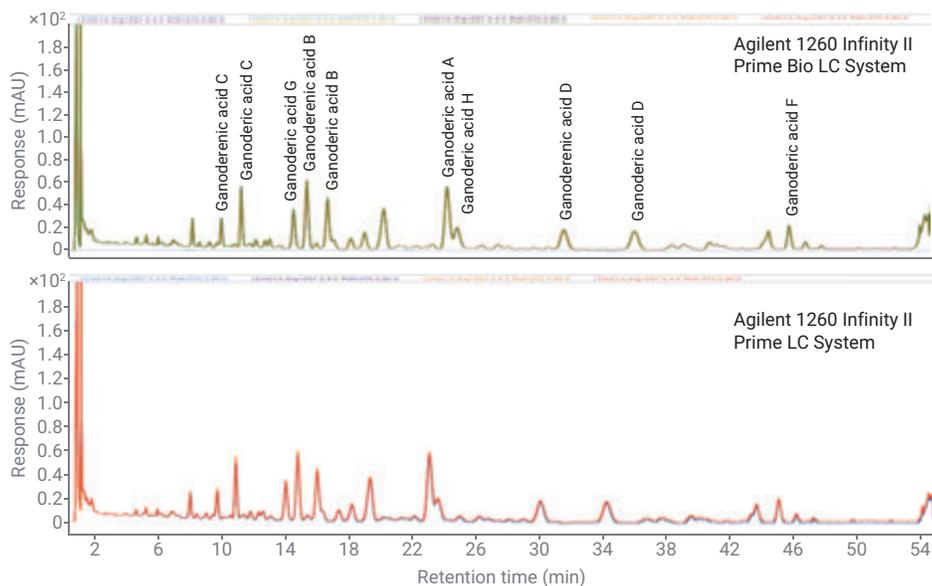
Compound ID RRLC Sample	Retention Time Agilent 1260 Infinity II Prime LC System (min)	RT %RSD	Retention Time Agilent 1260 Infinity II Prime Bio LC System (min)	RT %RSD	RT Deviation (min)	RT Deviation (%)
1	0.3604	0.5275	0.3593	0.0766	0.0012	-0.3233
2	0.8738	0.3954	0.8625	0.0578	0.0113	-1.2879
3	1.5150	0.3013	1.4900	0.0514	0.0250	-1.6513
4	2.0798	0.2418	2.0486	0.0252	0.0312	-1.5021
5	2.3447	0.1989	2.3117	0.0327	0.0331	-1.4106
6	2.5960	0.1765	2.5610	0.0238	0.0350	-1.3463
7	3.0619	0.1427	3.0236	0.0617	0.0384	-1.2531
8	3.4828	0.1268	3.4395	0.0684	0.0434	-1.2448
9	3.8720	0.1170	3.8227	0.0369	0.0492	-1.2719

### Shallow gradient method

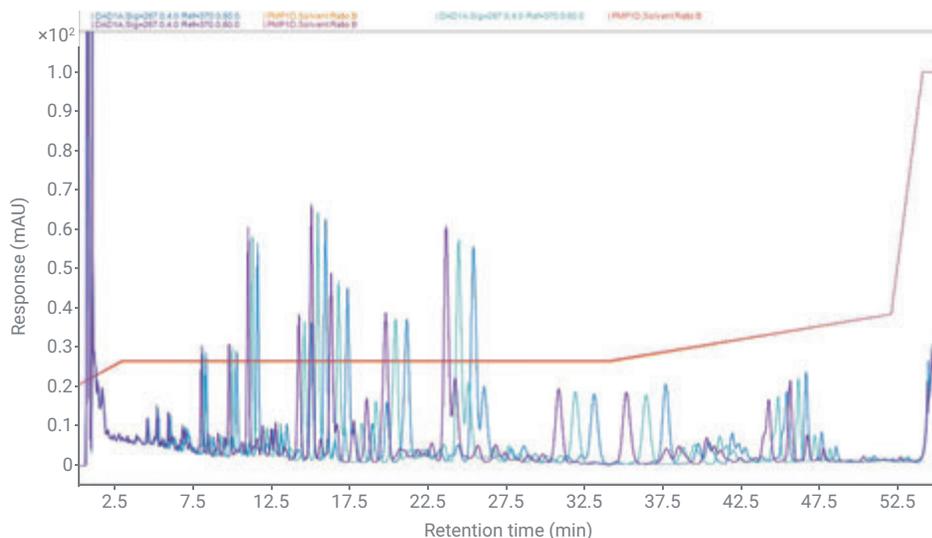
In the third application, a complex sample was chosen for analysis, which required a rather shallow gradient for separation. The Ganoderma Lucidum Fruiting Body Extract is a triterpenoid-enriched extract supplied by USP as a standard reagent (USP, Rockville, MD, USA). The separation method is based on the USP published chromatographic method<sup>1</sup>, and the assignment of the obtained peaks was done according to Ganoderma Lucidum Fruiting Body Dry Extract Lot Certificate F012B0, USP Catalog No. 1288372.<sup>2</sup> As presented in Figure 3, good mobile phase composition precision and an identical elution pattern of the sample were achieved with both systems.

However, a retention time shift can be observed, especially during the isocratic hold step during the method (Figure 3). This is further exhibited in Table 6, where the retention times of several peaks deviate more than 5%, indicating worse method compatibility for this particular method compared to the two former applications.

To further evaluate the root cause for the higher RT deviation, the method was slightly modified such that the isocratic hold step for B was adjusted to  $\pm 0.2\%$  of the original method. This simulated the change in retention time for the separated compounds due to slight composition accuracy differences between the two pumps, which were still performing within the specified range of  $\pm 0.4\%$ . As shown in Figure 4, this very small composition change resulted in a significant retention time shift of the eluting peaks, indicating that the method is too sensitive to composition changes and thereby overestimates the differences in composition accuracy between the two pumps. This effect generally needs to be considered when testing method compatibility.



**Figure 3.** Overlay of seven subsequent chromatograms of Ganoderma Lucidum Fruiting Body Extract separations on the Agilent 1260 Infinity II Prime LC System and Agilent 1260 Infinity II Prime Bio LC System. Assignment of peaks as mentioned above.



**Figure 4.** Effect of slight changes in isocratic hold step on retention time. Isocratic hold step was modified by  $\pm 0.2\%$  B. Results for the Agilent 1260 Infinity II Prime Bio LC System are shown. Green: 26.5% B isocratic hold step (original); blue: 26.3% B; magenta: 26.7% B.

**Table 6.** Calculation of retention time deviation between the Agilent 1260 Infinity II Prime LC System and Agilent 1260 Infinity II Prime Bio LC System. Retention times for the 1260 Infinity II Prime LC System act as a reference for calculating deviation. RT precision for each instrument is shown accordingly. Each RT and RT %RSD value is an average of seven measurements.

Compound ID Ganoderma L. Extract	Retention Time Agilent 1260 Infinity II Prime LC System (min)	RT %RSD	Retention Time Agilent 1260 Infinity II Prime Bio LC System (min)	RT %RSD2	RT Deviation (min)	RT Deviation (%)
1. Ganoderenic acid C	9.6596	0.1357	9.9924	0.0111	-0.3327	3.4446
2. Ganoderenic acid C	10.8330	0.1216	11.2505	0.0112	-0.4176	3.8546
3. Ganoderenic acid G	13.9869	0.0977	14.5709	0.0123	-0.5839	4.1749
4. Ganoderenic acid B	14.7444	0.0811	15.4070	0.0138	-0.6627	4.4943
5. Ganoderenic acid B	15.9688	0.0868	16.7174	0.0136	-0.7486	4.6878
6. Ganoderenic acid A	23.0435	0.1056	24.2967	0.0136	-1.2532	5.4386
7. Ganoderenic acid H	23.5823	0.1021	24.9284	0.0082	-1.3461	5.7080
8. Ganoderenic acid D	30.0558	0.0992	31.7005	0.0223	-1.6447	5.4721
9. Ganoderenic acid D	34.2341	0.1031	36.1752	0.0187	-1.9411	5.6700
10. Ganoderenic acid F	45.1035	0.0405	45.9687	0.0108	-0.8652	1.9182

## Conclusion

In this technical overview, the performance of the new Agilent 1260 Infinity II Prime Bio LC System was compared to the standard stainless steel-based Agilent 1260 Infinity II Prime LC System by running three different applications. With a high degree of reproducibility for each system for all three applications, good method compatibility could be demonstrated for the isocratic and fast gradient method. Retention time deviations remained below  $\pm 2\%$  relative to the retention times of the corresponding eluting compounds. The third application consisting of a shallow gradient, including a long-lasting isocratic hold step, gave higher RT deviations for a number of eluting compounds. It could be demonstrated

that the substantial deviation could derive from slight differences in mobile phase composition between the systems. Pumps that perform within their composition accuracy specifications can still reveal RT shifts larger than 5%, exhibiting the importance of a suitable method to be used for evaluating method compatibility.

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