

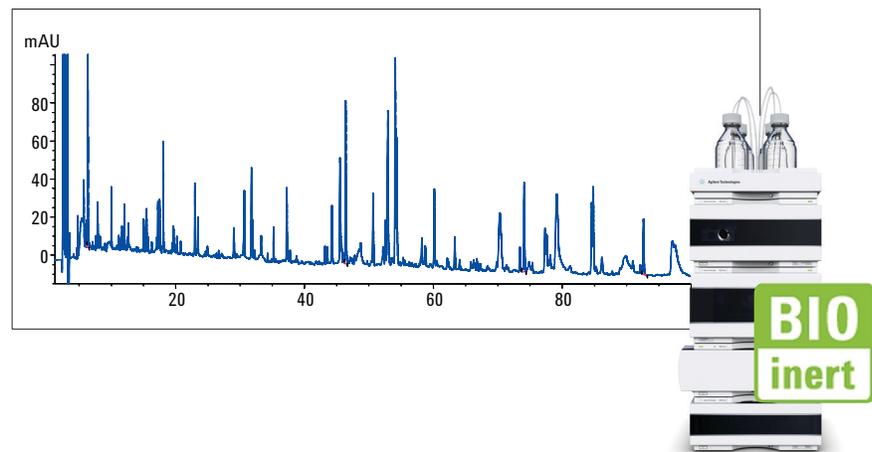
# QA/QC of monoclonal antibodies: High-resolution peptide mapping using the Agilent 1260 Infinity Bio-inert Quaternary LC and Agilent 1290 Infinity LC Systems

## Application Note

Biopharmaceuticals

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### Abstract

This Application Note describes an application solution for peptide mapping of a monoclonal antibody using the Agilent 1260 Infinity Bio-inert Quaternary LC System and proves the reliability of the solution. The bio-inertness and corrosion resistance of the instrument coupled with simple and reproducible methods make this solution particularly suitable for the QA/QC analysis of monoclonal antibody for the biopharmaceutical industry. To achieve even higher resolution as well as analytical sensitivity, we transferred the method to an Agilent 1290 Infinity LC System and sub-2- $\mu\text{m}$  particle columns. The power range of the 1290 Infinity LC System (pressure versus flow) overcomes the challenge of increased back pressure caused by smaller particles and longer columns making this system ideal for applications where high resolution and analytical sensitivity are a must.



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## Introduction

Peptide mapping is an essential technique for studying the primary structure of proteins. For recombinant protein pharmaceuticals, peptide mapping is used for the initial 'proof of structure' characterization, that is, to confirm expression of the desired amino acid sequence and to characterize any post-translational modifications. Further, peptide mapping is employed for subsequent lot-to-lot identity testing ('finger-printing') in support of bioprocess development and clinical trials. Peptide mapping is also used as the current method of choice for monitoring the 'genetic stability' of recombinant cell lines. Recently, peptide mapping has become a much more rapid and convenient method and has, therefore, expanded its utility for process monitoring and other higher volume applications<sup>1</sup>.

A significant challenge is the chromatographic separation of complex protein digests for the characterization of proteins. Here, we show the benefits of using an Agilent 1260 Infinity Bio-inert Quaternary LC System for resolving the peptides generated by tryptic digestion of IgG1 monoclonal antibody on an Agilent Poroshell 120 SB C18 4.6 × 150 mm, 2.7- $\mu$ m column. Method validation and robustness of an optimized RP-HPLC method for tryptic mapping of IgG1 are described for the 1260 Infinity Bio-inert Quaternary LC System. To take this a step further, the method was then transferred to a 1290 Infinity LC System with an Agilent ZORBAX RRHD SB C18, 2.1 × 150 mm, 1.8- $\mu$ m column. Ultrahigh performance liquid chromatography (UHPLC) separation using sub-2- $\mu$ m particles improves resolution per time and analytical sensitivity, shortens run times and

makes peptide mapping less challenging. Finally, two RRHD columns were coupled to evaluate longer sub-2- $\mu$ m particle columns for separating peptide maps.

## Equipment

### Instruments

A completely biocompatible Agilent 1260 Infinity Bio-inert Quaternary LC System with a maximum pressure of 600 bar consisting of the following modules was used:

- Agilent 1260 Infinity Bio-inert Quaternary LC Pump (G5611A)
- Agilent 1260 Infinity Bio-inert High Performance Autosampler (G5667A)
- Agilent 1200 Infinity Series Thermostat (G1330B)
- Agilent 1260 Infinity Thermostatted Column Compartment containing bio-inert click-in heating elements (G1316C, option 19)
- Agilent 1260 Infinity Diode Array Detector with 60-mm Max-Light high sensitivity flow cell (G4212B option 33)
- Agilent Poroshell 120 SB C18 column, 4.6 × 150 mm packed with 2.7- $\mu$ m particles (p/n 683975-902)

The complete sample flow path is free of any metal components such that the sample never gets in contact with metal surfaces. Solvent delivery is free of any stainless steel or iron components.

The UHPLC analysis was performed using the Agilent 1290 Infinity LC System with the following modules:

- Agilent 1290 Infinity Binary Pump with integrated vacuum degasser (G4220A) and 100- $\mu$ L Jet Weaver mixer
- Agilent 1290 Infinity High Performance Autosampler (G4226A)
- Agilent 1290 Infinity Thermostatted Column Compartment (G1316C)
- Agilent 1290 Infinity Diode Array Detector (G4212A) with Max-Light flow cell (1.0- $\mu$ L volume, 10-mm path length) (G4212-60008)
- Agilent ZORBAX RRHD SB C18 column, 2.1 × 150 mm, packed with 1.8- $\mu$ m particles (p/n 859700-902).

### Software

- Agilent ChemStation B.04.02

## Protein analysis

Protein analysis was done on the Agilent 2100 Bioanalyzer system with the Agilent Protein 230 kit. Protein loading and sample analysis were performed as described in the Protein 230 Kit Guide<sup>2</sup>. A 4- $\mu$ L solution of IgG1 and the digest was mixed with 2  $\mu$ L of sample buffer (non-reducing condition). The sample solution and ladder were placed at 95 °C for 5 minutes and further diluted with 84  $\mu$ L of water. Six microliters were applied to the protein chip for analysis. The Agilent 2100 Expert software was used for run control and data analysis.

## Chromatographic parameters

Chromatographic parameters used for reverse phase liquid chromatography and UHPLC are tabulated in Table 1.

## Reagents, samples and materials

Human monoclonal antibody IgG1 was a proprietary pharmaceutical molecule. DL-Dithiothreitol (DTT), iodoacetamide, trizma base, and ammonium carbonate were purchased from Sigma Aldrich. High quality sequence grade trypsin was from Agilent Technologies. All the chemicals and solvents used were HPLC grade and highly purified water from a Milli Q water purification system (Millipore Elix 10 model, USA) was used. Acetonitrile was of 'gradient grade' and purchased from Lab-Scan (Bangkok, Thailand).

| Parameter         | Agilent 1260 Infinity Bio-inert LC System   | Agilent 1290 Infinity LC System (single column)   | Agilent 1290 Infinity LC System (coupled column)  |
|-------------------|---|---|---|
| Column oven       | 50 °C   | 50 °C   | 50 °C   |
| Acquisition rate  | 20 Hz   | 20 Hz   | 20 Hz   |
| Data acquisition  | 214 and 280 nm  | 214 and 280 nm  | 214 and 280 nm  |
| Flow cell         | 60 mm path  | 10 mm path  | 10 mm path  |
| Injection volume  | 10 $\mu$ L (Needle with wash, flush port active for 6 seconds)  | 2.1 $\mu$ L (Needle with wash, flush port active for 6 seconds)   | 2.1 $\mu$ L (Needle with wash, flush port active for 6 seconds)   |
| Sample thermostat | 5 °C  | 5 °C  | 5 °C  |
| Mobile phase A    | Water + 0.1% TFA  | Water + 0.1% TFA  | Water + 0.1% TFA  |
| Mobile phase B    | Acetonitrile + 0.09% TFA  | Acetonitrile + 0.09% TFA  | Acetonitrile + 0.09% TFA  |
| Gradient          | At 0 min and 2% B<br>At 15 min and 20% B<br>At 50 min and 40% B<br>At 55 min and 60% B<br>At 60 min and 90% B<br>At 61 min and 2% B | At 0 min and 2% B<br>At 15 min and 20% B<br>At 50 min and 40% B<br>At 55 min and 60% B<br>At 60 min and 90% B<br>At 61 min and 2% B | At 0 min and 2% B<br>At 30 min and 20% B<br>At 100 min and 40% B<br>At 110 min and 60% B<br>At 120 min and 90% B<br>At 122 min and 2% B |
| Post run time     | 10 minutes  | 10 minutes  | 10 minutes  |
| Flow rate         | 1.2 mL/min  | 0.25 mL/min   | 0.25 mL/min   |

**Table 1**  
Chromatographic parameters.

## Procedure

### Digestion protocol

Tryptic digestion of IgG1 was carried out as described earlier<sup>3</sup>.

### Robustness study

The five critical method parameters listed below were varied to evaluate the robustness of the method

- Variation of injection volume ( $\pm 10\%$ )
- Variation of column temperature ( $\pm 5\%$ )
- Variation of gradient slope ( $\pm 10\%$ )
- Variation of modifier concentration ( $\pm 10\%$ )
- Variation of flow rate ( $\pm 2\%$ )

For each robustness parameter, 10  $\mu\text{L}$  of the digest were injected, six replicates were used to calculate area and RT. The percentage deviation (% accuracy) of area/retention time (RT) was calculated from the original method.

The peak width at 5 sigma for four arbitrarily chosen baseline separated peaks (six replicates) were averaged and these values were used for the calculation of peak capacity (Pc) according to the following formula:

$$Pc = 1 + (Tg/Pw)$$

Tg = gradient time

Pw = average peak width

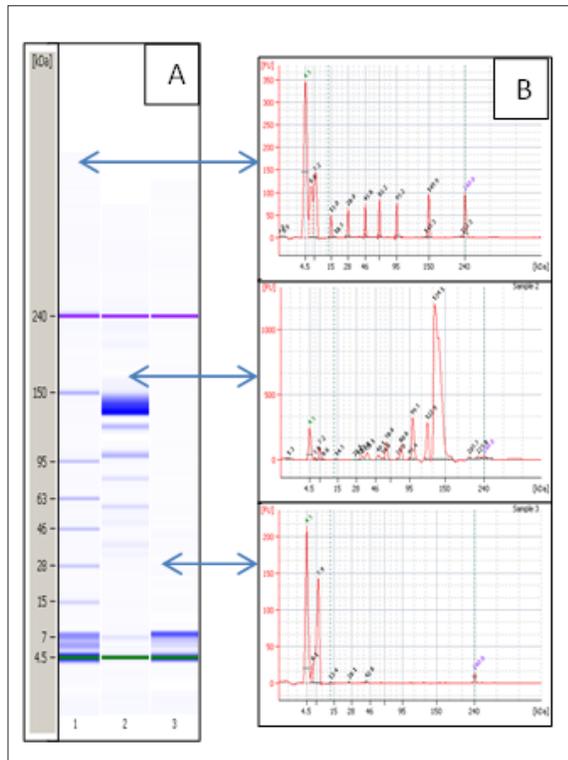
## Method transfer

The original method on the 1260 Infinity Bio-inert LC System was transferred to the 1290 Infinity LC System to showcase the performance (power range) of the 1290 Infinity LC System. The column used was an Agilent ZORBAX RRHD SB C18, 2.1  $\times$  150 mm, 1.8  $\mu\text{m}$ . The same gradient times and mobile phases of the 1260 Infinity Bio-inert LC System method was used. The Agilent Method Translator was used to determine the flow rate and injection volume. Further, two ZORBAX RRHD SB C18, 2.1  $\times$  150 mm, 1.8- $\mu\text{m}$  columns were coupled with gradient times doubled, keeping injection volume and flow rate unchanged. Peak capacities from 1260 Infinity Bio-inert LC, 1290 Infinity LC System with single and coupled columns were compared.

## Results and discussion

### Separation and detection

Trypsin is an endopeptidase that specifically cleaves peptide bonds on the carboxyl side of lysine and arginine residues in proteins. Complete digestion is a requirement for better separation of peptides by RP HPLC and the completeness of digestion determines the quality of the peptide mapping analysis. Here, we used the Agilent 2100 Bioanalyzer to qualify the efficiency of the digestion protocol. Comparison of undigested IgG1 (Lane 2) and digested IgG1 (Lane 3) clearly show the absence of a protein band in the digested sample demonstrating complete digestion of IgG1 (Figure 1).

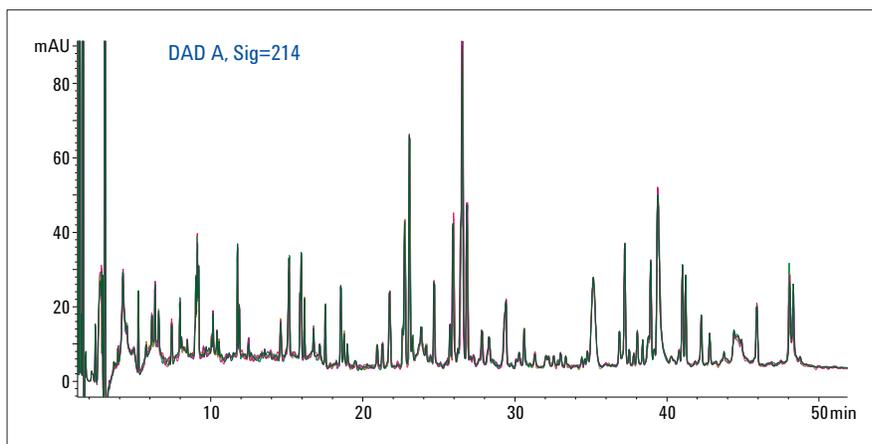


**Figure 1**  
Agilent 2100 Bioanalyzer system analysis of trypsin-digested and intact monoclonal IgG1 samples. Panel A. Gel like image, Lane 1, molecular weight marker, Lane 2, Intact IgG1, Lane 3, IgG1 digest. Panel B. Electropherogram. x-axis: relative size, y-axis: fluorescence signal intensity representing abundance.

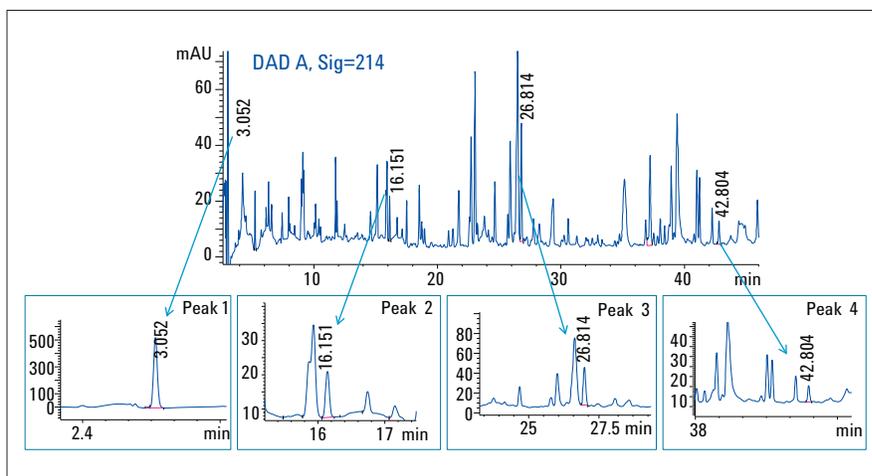
The peptide mapping analysis was initially performed on an Agilent 1260 Infinity Bio-inert Quarternary LC System to optimize the separation conditions (Table 1). The overlay of six replicates of an IgG1 digest results in sharp peaks with good resolution and excellent separation reproducibility (Figure 2). A representative chromatogram of the IgG1 digest (Figure 3) displays the four (baseline separated) peaks across the whole chromatogram which were selected for method validation and peak capacity calculation.

### Precision of retention time and area

Average retention times and area RSDs for the four selected peaks from six replicates of an IgG1 peptide map are summarized in Table 2. The retention time RSDs for these peaks were less than 0.05% for peaks with higher  $k'$  (peak 2–4) demonstrating excellent gradient reproducibility. Even the very early eluting peak 1 showed retention time RSD at 0.26%. Peak area RSDs were less than 2.14% indicating precise sample injection. The RSD values demonstrate the robustness of the method and thus the precision of the LC system.



**Figure 2**  
Overlay of six replicates of an IgG1 digest separation on an Agilent 1260 Infinity Bio-inert Quarternary LC System using an Agilent Poroshell 120 SB C18 4.6 × 150 mm, 2.7- $\mu$ m column.



**Figure 3**  
Peaks selected for method validation studies and peak capacity calculation.

| Peak no. | Retention time |         | Peak area      |         |
|----------|----------------|---------|----------------|---------|
|          | Mean (min)     | RSD (%) | Mean (mAU/min) | RSD (%) |
| 1        | 3.03           | 0.26    | 1393           | 0.40    |
| 2        | 16.16          | 0.04    | 63.98          | 2.14    |
| 3        | 26.86          | 0.04    | 250.83         | 0.63    |
| 4        | 42.80          | 0.01    | 54.33          | 1.90    |

**Table 2**  
Retention time and area RSD (%), n = 6 for four selected peaks across whole chromatogram.

## Robustness

To evaluate the robustness of the method, six critical method parameters of the original method were varied (Tables 3 and 4). The impact of injection volume, column temperature, gradient slope, modifier concentration and flow rate on retention time was within the acceptable limits. Allowed deviations for retention time and area were set to  $\pm 3.0\%$  (Table 3) and  $\pm 5\%$  (Table 4) respectively. The red numbers indicate where the result exceeded the allowed deviation. When the injection volume was varied by  $\pm 10\%$  compared to the actual method, the area RSD of all four peaks deviated more than the allowed limit. A variation in flow rate causes the area RSD of peak 2 and peak 3 to deviate marginally and that of peak 4 to deviate significantly. Our results show that the method is reliable for routine QA/QC application. To a great extent, the performance remains unaffected by variations in critical parameters. However, some parameters are critical and must be carefully controlled.

## Peak capacity

Peak capacity essentially is the number of equally well-resolved peaks that can be fit into a chromatogram between the holdup volume and upper limit in retention. The peak capacity values calculated for an Agilent Poroshell 120 SB C18 4.6  $\times$  150 mm, 2.7- $\mu$ m column are shown in Table 5. The peak capacity value of 292 is appropriate for peptide mapping applications.

| Parameters   | Variations  | RT deviation (limit : $\pm 3.0\%$ ) |        |        |        |
|--|-------------|-------------------------------------|--------|--------|--------|
|  |             | Peak 1                              | Peak 2 | Peak 3 | Peak 4 |
| Variation in injection volume (10 $\mu$ L $\pm 10\%$ ) | - 1 $\mu$ L | -0.098                              | 0.006  | -0.11  | 0.10   |
|  | +1 $\mu$ L  | -0.098                              | 0.12   | 0.07   | 0.13   |
| Variation in column temperature ( $\pm 5\%$ )          | - 5%        | -0.69                               | 0.30   | 0.14   | 0.26   |
|  | +5%         | 0.55                                | 0.50   | 0.44   | 0.32   |
| Variation in gradient slope ( $\pm 10\%$ )             | - 10%       | -0.098                              | 1.32   | 2.19   | 0.83   |
|  | +10%        | -0.42                               | 0.06   | 0.81   | 0.69   |
| Variation in modifier concentration ( $\pm 10\%$ )     | -10%        | 0.0                                 | 0.87   | 0.85   | 0.241  |
|  | +10%        | -0.72                               | 0.68   | 0.14   | 0.02   |
| Variation of flow rate ( $\pm 2\%$ )                   | - 2%        | -1.40                               | 0.99   | 1.19   | 0      |
|  | + 2%        | 1.87                                | 0.56   | 0.37   | 0.26   |

**Table 3**  
Robustness (RT RSD %) n = 6, RT deviation limit  $\pm 3\%$ .

| Parameters   | Variations  | Area deviation (limit : $\pm 5.0\%$ ) |        |        |        |
|--|-------------|---------------------------------------|--------|--------|--------|
|  |             | Peak 1                                | Peak 2 | Peak 3 | Peak 4 |
| Variation in injection volume (10 $\mu$ L $\pm 10\%$ ) | - 1 $\mu$ L | 11.77                                 | 17.05  | 4      | 3.84   |
|  | +1 $\mu$ L  | -11.19                                | -14.24 | -11.6  | -9.61  |
| Variation in column temperature ( $\pm 5\%$ )          | - 5%        | -1.65                                 | -3.26  | 3.2    | -3.84  |
|  | +5%         | -0.71                                 | -0.71  | 1.7    | -5.76  |
| Variation in gradient slope ( $\pm 10\%$ )             | - 10%       | -2.22                                 | 2.22   | 0.40   | 1.92   |
|  | +10%        | -3.08                                 | -3.086 | 2.4    | 7.6    |
| Variation in modifier concentration ( $\pm 10\%$ )     | -10%        | 3.87                                  | 2.97   | -5.2   | -1.9   |
|  | +10%        | -0.78                                 | -0.15  | -1.44  | -3.84  |
| Variation of flow rate ( $\pm 2\%$ )                   | - 2%        | -4.73                                 | 1.40   | -5.6   | -7.6   |
|  | + 2%        | 0                                     | 7.19   | -2.4   | -11.53 |

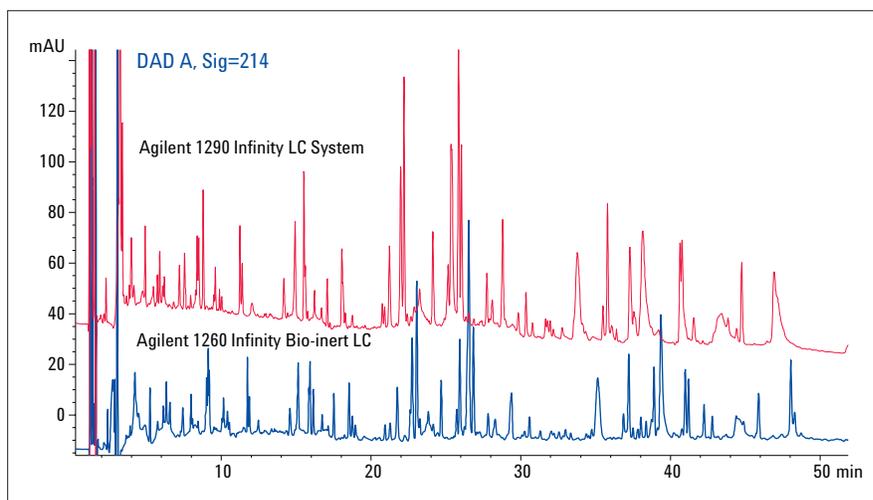
**Table 4**  
Robustness (area RSD %) n = 6, Area deviation limit  $\pm 5\%$ .

| 5 sigma peak width          | Peak 1 | Peak 2 | Peak 3 | Peak 4 |
|-----------------------------|--------|--------|--------|--------|
| Replicate 1                 | 0.09   | 0.132  | 0.22   | 0.225  |
| Replicate 2                 | 0.095  | 0.153  | 0.22   | 0.237  |
| Replicate 3                 | 0.092  | 0.265  | 0.2    | 0.245  |
| Replicate 4                 | 0.1    | 0.132  | 0.19   | 0.246  |
| Replicate 5                 | 0.095  | 0.131  | 0.21   | 0.288  |
| Replicate 6                 | 0.081  | 0.132  | 0.19   | 0.288  |
| Avg                         | 0.0921 | 0.140  | 0.205  | 0.254  |
| Avg 5 sigma peak width (Pw) |        |        | 0.172  |        |
| Gradient time (Tg)          |        |        | 50     |        |
| Tg/Pw                       |        |        | 291    |        |
| Peak capacity = 1 + (Tg/Pw) |        |        | 292    |        |

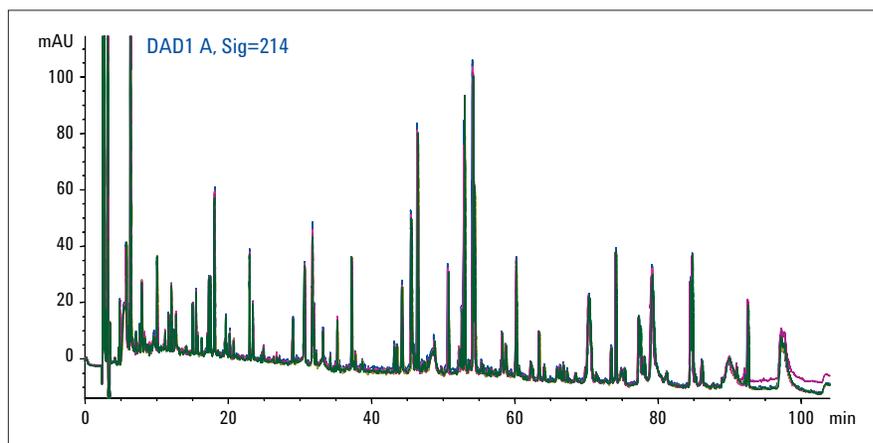
**Table 5**  
Peak capacity (for four selected baseline separated peaks) n = 6.

## Method transfer to the 1290 Infinity LC System

The 1290 Infinity LC System offers an exceptional power range for separating complex samples. Hence, the method was transferred to the 1290 Infinity LC System using the method translator. Figure 4 shows the overlay of peptide maps of IgG1 on 1260 Infinity Bio-inert LC System with Poroshell 120 SB C18 and 1290 Infinity LC System with a ZORBAX RRHD SB C18, 2.1 × 150 mm, 1.8- $\mu$ m column. The two chromatograms are highly similar. Figure 5 shows an overlay of six injections of the IgG1 digest analyzed on the 1290 Infinity LC System with two coupled RRHD columns. The 1290 Infinity LC System with a single or two coupled narrow bore columns produced sharper and narrower peaks as compared to 1260 Infinity Bio-inert LC System as indicated by an increased peak capacity (Table 6). Peak capacity increased 7% with single RRHD column and 39% with coupled columns indicating an exceptional ability for separating complex samples. Hence, the usage of 1.8- $\mu$ m columns results in superior resolution at similar separation time.



**Figure 4**  
Peptide map of IgG1. Overlay of chromatograms from an Agilent 1260 Infinity Bio-inert LC System with an Agilent Poroshell 120 SB C18 4.6 × 150 mm, 2.7  $\mu$ m column and an Agilent 1290 Infinity LC System with a single Agilent ZORBAX RRHD SB C18, 2.1 × 150 mm, 1.8- $\mu$ m column.



**Figure 5**  
Overlay of six replicate injections of IgG1 digest on an Agilent 1290 Infinity LC System using coupled columns (Agilent ZORBAX RRHD SB C18, 2.1 × 150 mm, 1.8- $\mu$ m columns).

| Instrumentation platform           | Column   | Peak capacity |
|------------------------------------|--|---------------|
| Agilent 1260 Infinity Bio-inert LC | Agilent Poroshell 120 SB C18<br>4.6 x 150 mm, 2.7 $\mu$ m        | 292           |
| Agilent 1290 Infinity LC           | Single Agilent ZORBAX RRHD SB C18,<br>2.1 x 150 mm, 1.8 $\mu$ m  | 313           |
| Agilent 1290 Infinity LC           | Coupled Agilent ZORBAX RRHD SB C18,<br>2.1 x 150 mm, 1.8 $\mu$ m | 406           |

**Table 6**  
Peak capacity comparison (average of six replicates).

## Conclusion

Peptide mapping is an important approach for studying the primary structure of proteins and is widely applied in biopharmaceutical QA/QC. Here, we showcase several Agilent tools for the analysis of monoclonal antibodies with this technique. We first used the Agilent 1260 Infinity Bio-inert Quarternary LC System to develop a simple and high resolution separation of a IgG1 tryptic digest using a Poroshell 120 SB C18 4.6 × 150 mm, 2.7- $\mu$ m column. Area, RT precision and robustness of the method were excellent and show the reliability of the method. To achieve even higher peak capacity, the original method was transferred to an Agilent 1290 Infinity UHPLC system with ZORBAX RRHD SB C18, 2.1 × 150 mm, 1.8- $\mu$ m columns. Using 1.8- $\mu$ m columns results in superior resolution as indicated by the increased peak capacity. Peak capacity can be increased further significantly by coupling two 1.8- $\mu$ m columns. This is only possible with systems capable to tolerate ultra-high backpressures. Additionally, the Agilent 2100 Bioanalyzer system provides certainty regarding the efficiency of the digestion protocol.

## References

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