



# Innovator and Biosimilar Monoclonal Antibody-Peptide Map Comparison Using the Agilent 7100 Capillary Electrophoresis System and Agilent MatchCompare Software

## Application Note

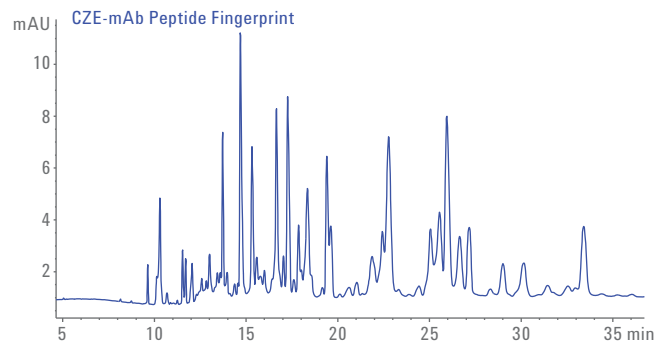
Biopharmaceutical

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### Abstract

Capillary electrophoresis (CE) has been used in the biopharmaceutical industry for analysis of monoclonal antibodies (mAb) in the forms of capillary zone electrophoresis, capillary isoelectric focusing, and capillary gel electrophoresis. This Application Note presents the use of the Agilent 7100 Capillary Electrophoresis system for peptide digest analysis of innovator and biosimilar rituximab. Peptide digest is generally used to evaluate the comparability of two samples. In this study, high separation efficiency was achieved using bare fused silica capillary followed by UV detection. The peptide map was then analyzed using Agilent MatchCompare software to assess the similarities.



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## Introduction

Characterization of post-translational modifications (PTMs) in a monoclonal antibody (mAb) has been extensively reported, owing to their pharmaceutical importance. PTM often leads to heterogeneity within mAbs, which can severely affect efficacy and quality<sup>1</sup>. Several analytical methods are used to characterize mAbs, of which capillary electrophoresis (CE) is a common and well-established method<sup>2</sup>. CE is a versatile analytical technique offering several advantages, including different separation modes within the same instrument, low sample and solvent consumption, and the flexibility to use different detection techniques.

This Application Note demonstrates the use of an Agilent 7100 Capillary Electrophoresis system for peptide analysis of innovator rituximab and biosimilar rituximab samples followed by data analysis using Agilent MatchCompare software.

## Experimental

### Materials and instrumentation

Ammonium bicarbonate, acetylated trypsin, urea, dithiothreitol (DTT), iodoacetamide (IAA), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich. Phosphate buffer pH 2.5 supplemented with 0.05 % hydroxyl ethyl cellulose, 0.1 N NaOH, and 1 N NaOH were purchased from Agilent Technologies. Innovator and biosimilar rituximab were purchased from a local pharmacy. The samples were made to a stock solution of 10 mg/mL with water. An Agilent 7100 Capillary Electrophoresis system (G7100A) was obtained from Agilent Technologies.

### Sample preparation

Innovator rituximab and biosimilar rituximab were reconstituted in 6 M urea in 25 mM ammonium bicarbonate, pH 8.0. The samples were denatured by adding 10 mM DTT (final), and incubated for 1 hour at 37 °C. The reduced cysteines were then alkylated by adding 40 mM of iodoacetamide (final), and incubated in the dark for 1 hour at 37 °C. After alkylation, excess iodoacetamide was then quenched by adding DTT. Trypsin in a 1:20 ratio was added to the proteins, and incubated overnight at 37 °C. Following the overnight incubation, the digestion was stopped by acidification of the sample using TFA. The peptides were then desalted using ZipTipC18, and analyzed by CE, or stored at –80 °C.

### CE analysis

The samples were analyzed in capillary zone electrophoresis (CZE) mode using a bare fused-silica capillary with a total length of 65 cm and an effective length of 56 cm. A new capillary was prepared by sequential 10-minute flushes with acetonitrile, 0.1 N HCl, 1 N NaOH, and water, followed by a 20-minute flush with BGE. Once a day, capillaries were conditioned as follows: flush with 0.1 N NaOH for 30 minutes, with water for 15 minutes, and with BGE for 30 minutes and voltage equilibration at 25 kV for 3 minutes. Prior to every run, capillaries were conditioned by flushing with BGE for 10 minutes. The samples were hydrodynamically injected for 25 seconds at 50 mbar. Table 1 presents the conditions used for the CE.

Table 1. CE parameters used in the study.

Parameter	Value
Capillary	Bare fused-silica, total length of 65 cm with effective length of 56 cm, 50 µm id
Sample	Innovator rituximab and biosimilar rituximab
Injection	25 seconds at 50 mbar
Buffer	50 mM Phosphate buffer pH 2.5 + 0.05 % hydroxyl ethyl cellulose
Voltage	+25 kV
Temperature	25 °C
DAD	200 nm/16 nm bandwidth, 214 nm/4 nm bandwidth

### Data analysis

Agilent OpenLAB CDS ChemStation (C.01.07) Software was used for data acquisition and data analysis. Agilent MatchCompare (A.01.02) was used to compare the chromatograms and to obtain the similarity scores<sup>3</sup>.

## Results and Discussion

### CE Peptide analysis

The separation of the peptides was achieved using a 50 mM phosphate buffer pH 2.5, supplemented with 0.05 % hydroxyl ethyl cellulose over a run time of 40 minutes. A good separation of the peptides was achieved with approximately 60 peaks resolved between migration times of 10 to 35 minutes. Figure 1 shows the peptide map of the innovator and biosimilar rituximab.

PTMs can alter the charges on the peptide molecule and, therefore, the peptide separation profile. CZE offers an advantage over other CE modes as the separation is based on the charge-to-mass ratio of the analyte<sup>4</sup>. The overlaid separation profile of the innovator and biosimilar presented in Figure 2 shows the differences in the peptide map. Variations include the higher intensity of a few peptide peaks in the biosimilar. These variations imply modification in the biosimilar that could arise from charge variants or any other PTMs. These differences could indicate a significant change to the antibody's physical properties or efficacy.

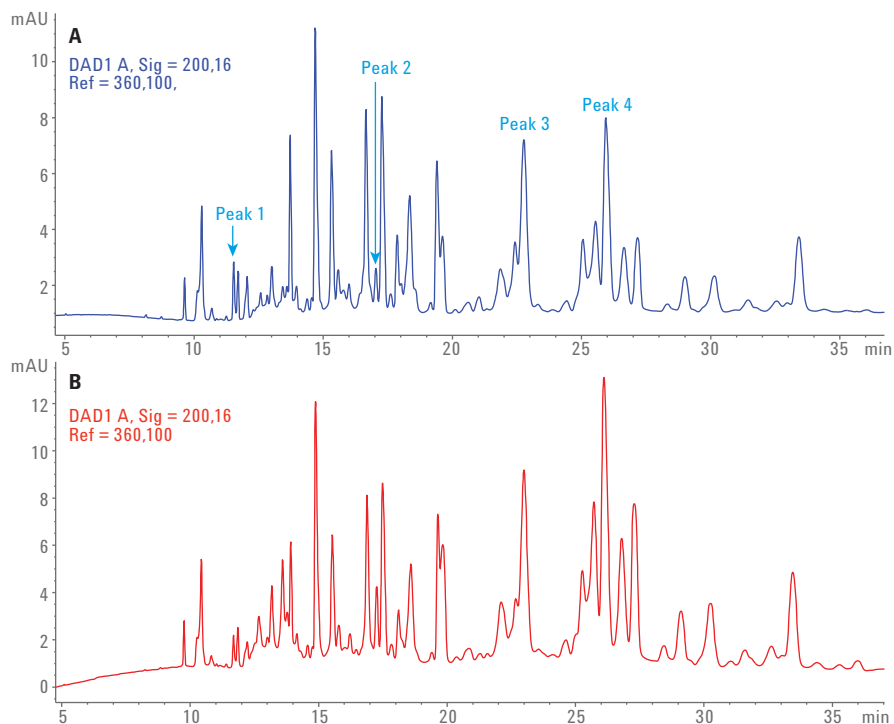


Figure 1. Peptide map of innovator rituximab (A) and biosimilar rituximab (B). Four peaks selected for the reproducibility assessment are indicated in A.

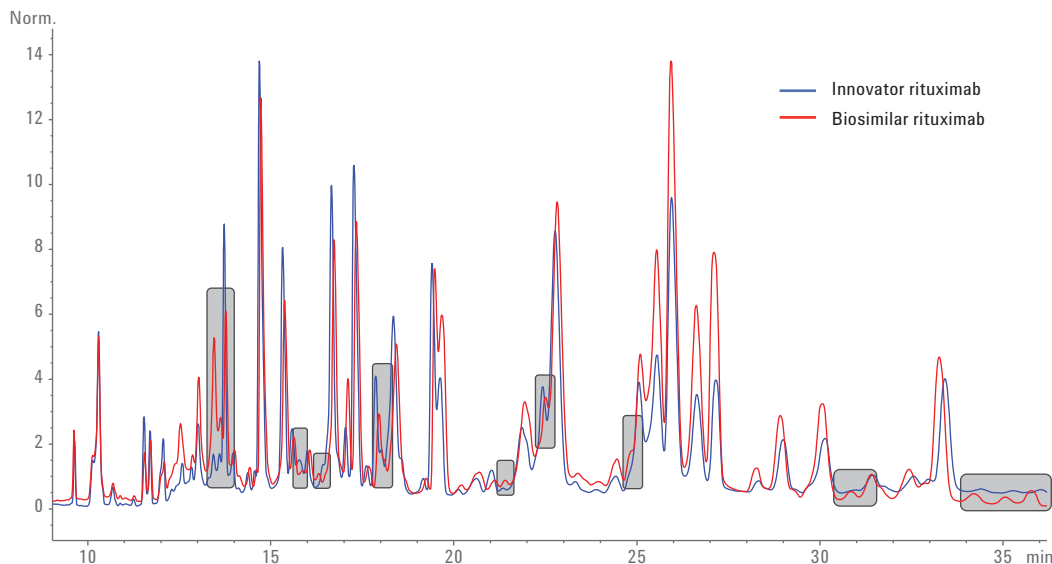


Figure 2. Zoomed view of the separation profile overlay highlighting the differences in the separation profile.

A cation exchange chromatography study on the same samples also suggested the presence of charge variants in the biosimilar<sup>5</sup>. Another study involving mass spectrometry analysis of the same samples showed post-translation modifications such as lysine truncation, oxidation, and deamidation in the biosimilar samples<sup>6</sup>. CE offers the advantage of separating small and highly charged peptides, which might be a challenge in LC separation<sup>7</sup>. CE with a downstream mass spectrometry analysis would help in further characterization, and can be used as a complementary technique for the analysis of biopharmaceuticals as presented earlier<sup>8</sup>.

The reproducibility of the CE separation was assessed by calculating the RSD for the migration and peak area of four peaks identified in Figure 1. The migration time RSDs for both samples were 2.1 % or better, and peak area RSDs were 12.1 % or better suggesting good separation reproducibility.

### Agilent MatchCompare

Comparing two peptide mapping profiles requires a manual comparison of each peak between the two profiles for migration time, peak area, peak shape, and other characteristics. This time-consuming activity is prone to human errors, requires expert experience, and is a challenge in a manufacturing scenario where several batches per day have to be monitored.

Agilent OpenLAB MatchCompare is an add-on tool for Agilent ChemStation that automates the comparison of two chromatograms<sup>9</sup>. The tool compares a sample chromatogram against a reference chromatogram to match identical peaks with adjustment for migration shifts. Peaks are matched, then the peak area percent is calculated and checked against predefined/user-defined

Table 2. Migration time and peak area reproducibility (n = 5).

Peaks	Migration time (min)				Peak area			
	Innovator rituximab		Biosimilar rituximab		Innovator rituximab		Biosimilar rituximab	
	Mean	RSD %	Mean	RSD %	Mean	RSD %	Mean	RSD %
Peak 1	11.6	1	11.6	0.7	9.2	10.4	7.1	4.3
Peak 2	17.2	1.7	17.1	0.7	12.9	8.5	30.4	10.1
Peak 3	23.1	1.9	22.7	0.7	108.9	5.4	133.8	11.2
Peak 4	26.3	2.1	25.9	0.6	112.5	4	169.4	12.1

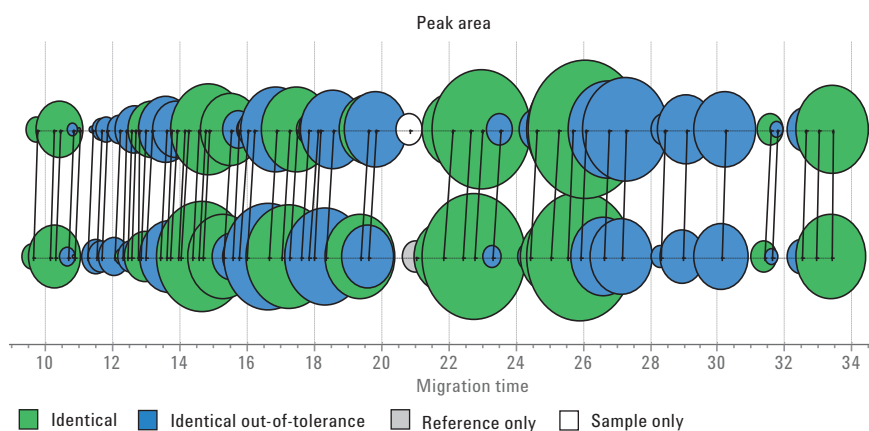


Figure 3. Agilent MatchCompare similarity graph showing sample peptide fingerprint (above) matched with reference fingerprint (below).

tolerance limits (temporal tolerance = 0.2 minutes; initial shift = 0.165 minutes) for peak area. The tool then presents a color-coded graphic to indicate identical peaks (green), identical peaks with out-of-tolerance limits (blue), and unique peaks within the sample or reference (white or gray).

The CZE peptide maps of innovator and biosimilar rituximab were analyzed using the MatchCompare software, giving a similarity score of 0.9782. The minor differences presented in Figure 2 contributed to the score calculation.

MatchCompare automates the peptide map comparison, presents an objective report on similarities of the samples, and highlights any differences. This provides an excellent solution for comparison studies such as innovator-biosimilar characterization and batch-to-batch quality control.

## Conclusion

- CZE is a suitable technique for peptide mapping to study heterogeneity differences between innovator and biosimilar monoclonal antibodies.
- The Agilent 7100 Capillary Electrophoresis system shows excellent separation of peptides on bare fused-silica capillaries, with a migration time repeatability of 2.1 % RSD or better, and a peak area repeatability of 12.1 % RSD or better.
- Agilent MatchCompare software is a useful tool for automatic and objective comparison of peptide maps.
- In agreement with LC and LC/MS data, the present study shows significant differences between innovator and biosimilar rituximab.

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