Workflow Solution for the Characterization of Biosimilar using Different Modes of Analytical Chromatography Techniques

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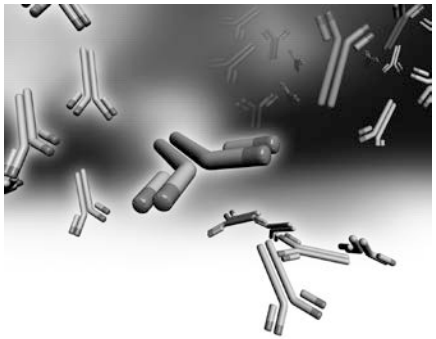


Introduction

- In 2010, Congress implemented a shortened licensure path for biological products that are proven to be biosimilar to an FDA-approved biological product.

- Section 351(i) defines biosimilarity to mean “that the biological product is highly similar to the reference product notwithstanding minor differences in clinically inactive components” and that “there are no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency of the product.”

- The FDA recommends that the analytical similarity evaluation originates with an understanding of the structural/physicochemical and functional characteristics of the innovator reference product. The comprehensive use of various modes of analytical chromatography is useful in this regard[1].

Introduction (cont.)

Analytical high performance liquid chromatography (HPLC) techniques that are used in comparing the similarity between biosimilars and innovator reference products include:

- Size Exclusion Chromatography
  - Purity study – aggregate and fragment content
  - Stability study
- Reversed Phase Chromatography
  - Peptide analysis
- HILIC Chromatography
  - Orthogonal verification
- Hydrophobic Interaction Chromatography
  - Impurity analysis (hydrophobicity)
- Ion Exchange Chromatography
  - Charged isoform analysis

Size Exclusion Chromatography

- Size exclusion chromatography (SEC) is the method of choice for purity analysis and detecting aggregates of drug product.
- SEC can be utilized to compare the presence of aggregates and fragments in the innovator and reference product and to confirm or disprove equivalent hydronamic radii between the two molecules.

Size Exclusion Chromatography

Materials and Methods

Column: TSKgel® UP-SW3000, 2 µm, 4.6 mm ID × 30 cm

Table 1: Column Characteristics

Column size	4.6 mm ID × 30 cm
Base material	Silica
Stationary phase	Diol
Particle size	2 µm
Pore size	25 nm
Exclusion limit (Proteins)	800 kDa
Separation range (Proteins)	10 - 500 kDa

Size Exclusion Chromatography

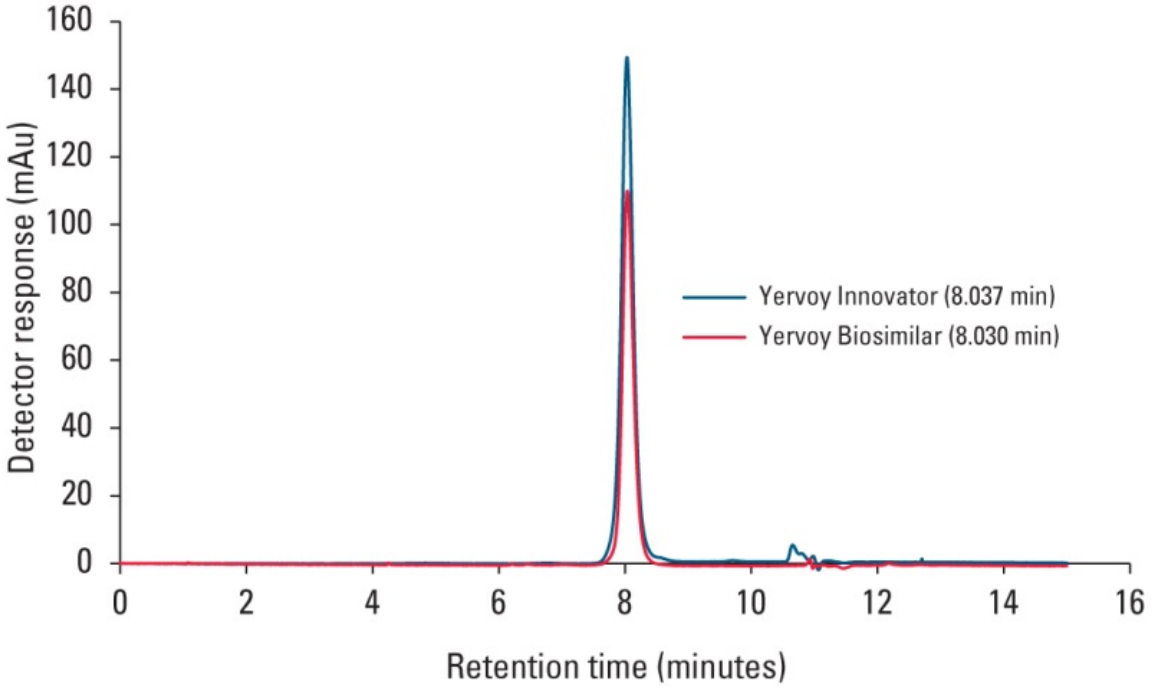
Materials and Methods (cont.)

Instrument:	Thermo Fisher Dionex Ultimate® 3000 with Chromeleon® v. 6.8
Mobile phase:	100 mmol/L KH_2PO_4/Na_2HPO_4, pH 6.7, 100 mmol/L Na_2SO_4, 0.05% NaN_3
Flow rate:	0.35 mL/min
Pressure:	28.5 MPa
Detection:	UV @ 280 nm
Temperature:	25º C
Injection vol.:	5 µL unless stated
Samples:	Humira® Innovator (5 mg/mL) Humira Biosimilar (4 mg/mL) Yervoy® Innovator (5 mg/mL) Yervoy Biosmilar (3.7 mg/mL)

Size Exclusion Chromatography

Results

Figure 1: HPLC Analysis of Yervoy Innovator and its Biosimilar Using TSKgel UP-SW3000

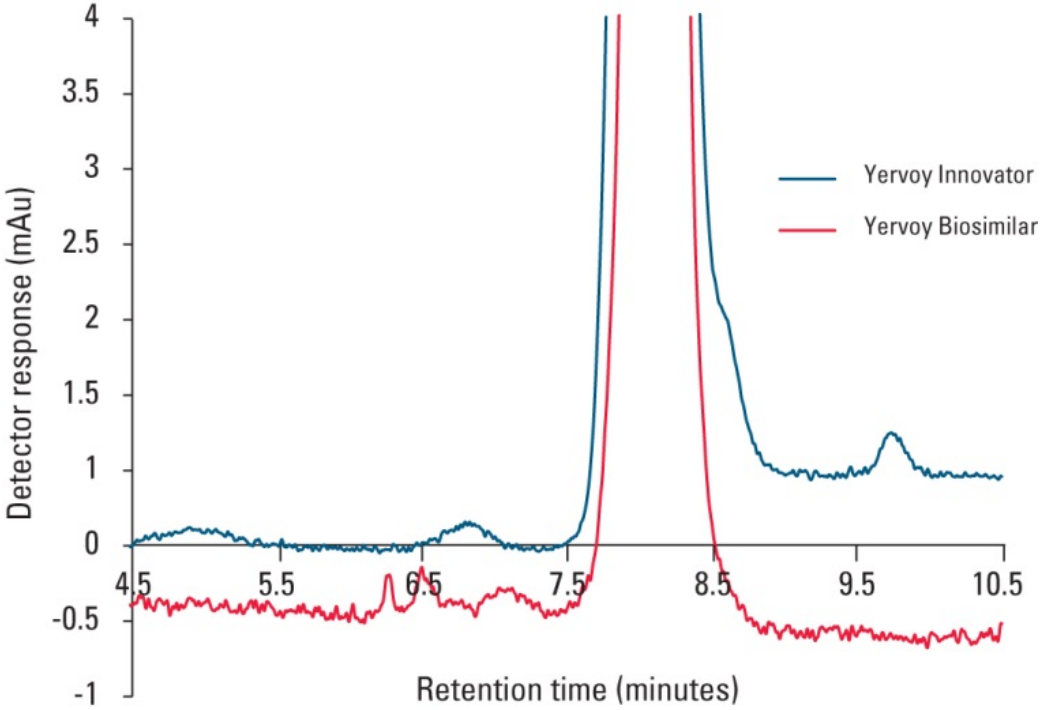


- The Yervoy innovator and biosimilar exhibit similar retention times.
- 6 consecutive injections yielded low % RSD for the peak parameters such as retention time, peak asymmetry and theoretical plates[2].

Size Exclusion Chromatography

Results (cont.)

Figure 2: HPLC Analysis of Yervoy Innovator and its Biosimilar Using TSKgel UP-SW3000: Magnified View



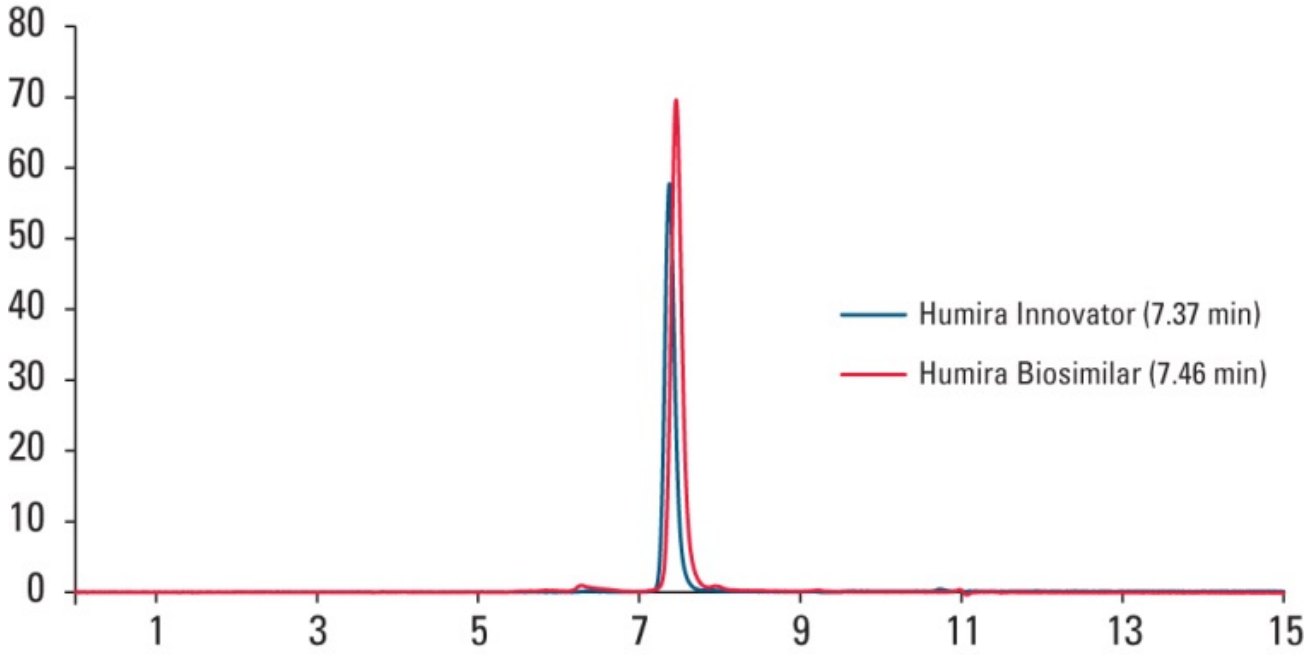
Retention time (minutes)				
	HMW	Dimer	Monomer	Fragment
Yervoy Innovator	4.837	6.780	8.030	9.707
Yervoy Biosimilar	6.447, 6.223	6.987	8.037	-
% Peak area (mAU*min)				
	HMW	Dimer	Monomer	Fragment
Yervoy Innovator	0.18	0.20	99.47	0.15
Yervoy Biosimilar	0.26	0.23	99.56	0

The zoomed-in profile provides a closer look at the baseline and impurities present in each sample. Both the innovator and biosimlar exhibit >99% purity.

Size Exclusion Chromatography

Results (cont.)

Figure 3: HPLC Analysis of Humira Innovator and its Biosimilar Using TSKgel UP-SW3000

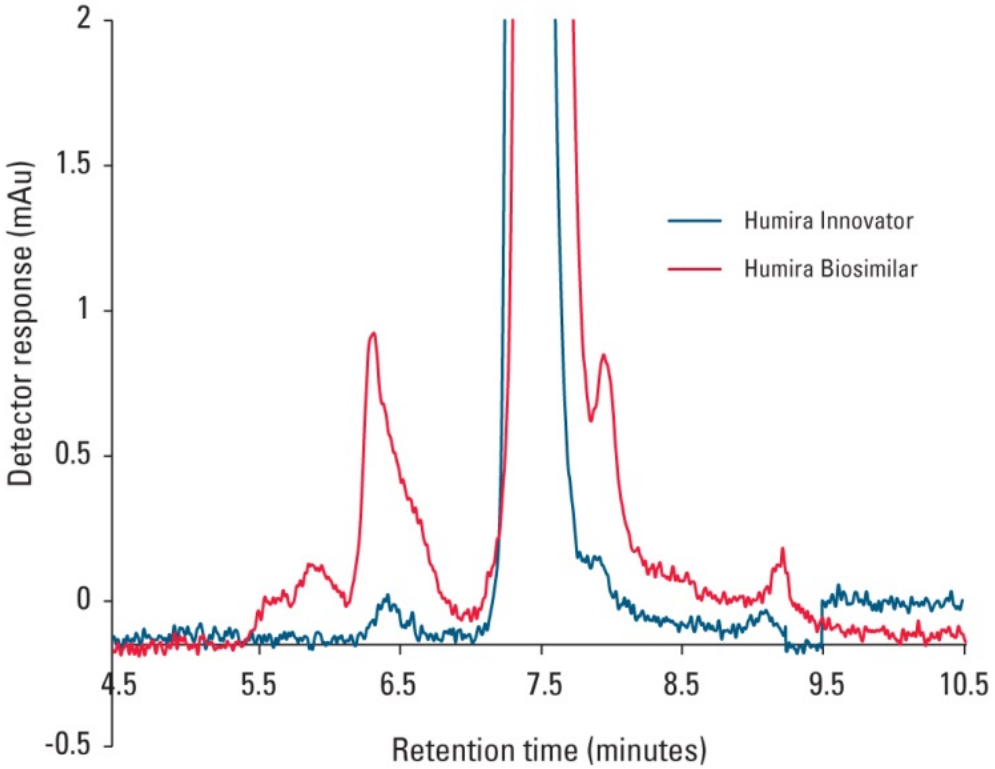


The Humira innovator and biosimilar show slight variation in retention time.

Size Exclusion Chromatography

Results (cont.)

Figure 4: HPLC Analysis of Humira Innovator and its Biosimilar Using TSKgel UP-SW3000: Magnified View



Retention time (minutes)				
	HMW	Dimer	Monomer	Fragment
Humira Innovator	0	6.387	7.37	7.883, 9.097
Humira Biosimilar	5.843	6.283	7.46	7.960, 9.217
% Peak area (mAU*min)				
	HMW	Dimer	Monomer	Fragment
Humira Innovator	0	0.27	99.59	0.14
Humira Biosimilar	0.15	1.73	97.66	0.46

The zoomed-in profile provides a closer look at the baseline and impurities present in each sample. A higher percentage of aggregates and fragments are noted in the biosimilar sample.

Reversed Phase Chromatography

Reversed phase chromatography/mass spectrometry (RPC/MS) is a powerful technique that can be used to compare the peptide sequences that are present in the innovator and biosimilar.

Reversed Phase Chromatography

Materials and Methods

Column: TSKgel ODS-100V, 5 µm, 4.6 mm ID × 15 cm

Table 2: Column Characteristics

Pore size (mean):	10 nm
Molar mass limit:	1.0×10^4 Da
Endcapped:	Yes
Particle size:	5 µm
pH stability:	2.0 - 7.5
Functional group:	octadecylmethylsilane
% carbon:	15
Surface area (m^2/g):	450

Reversed Phase Chromatography

Materials and Methods (cont.)

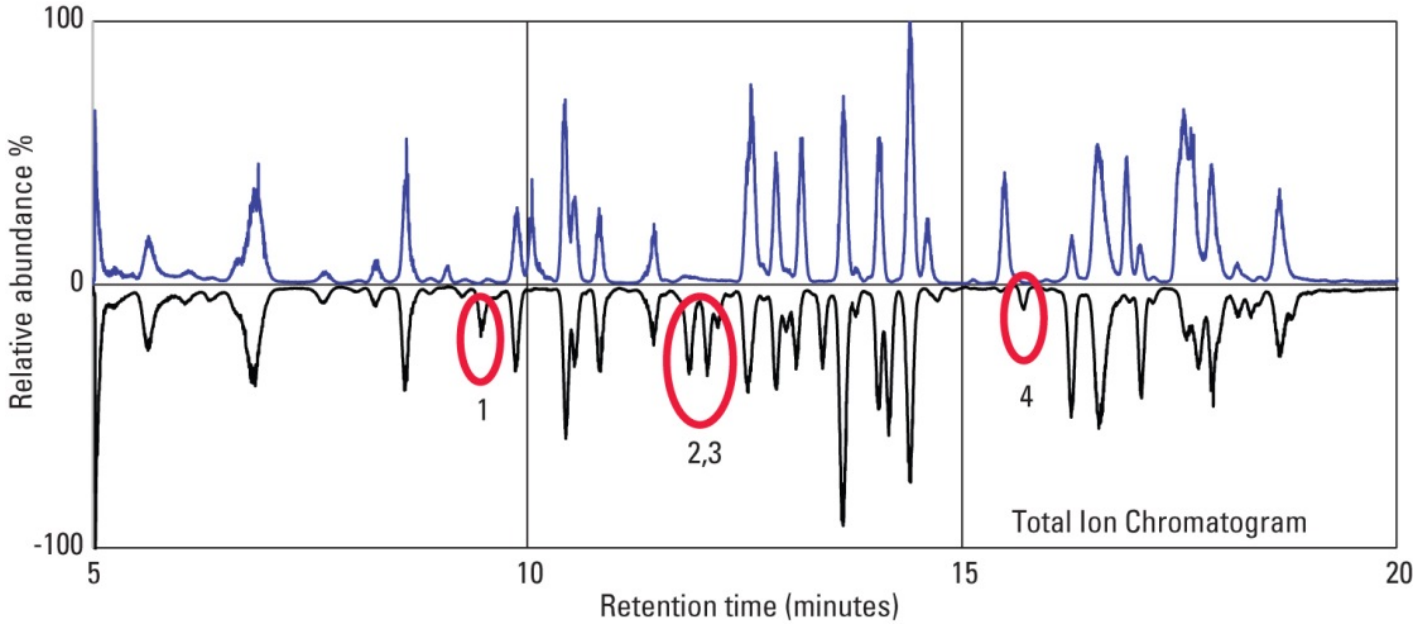
Instrument:	Thermo Orbitrap Q Exactive™ coupled with Thermo Ultimate 3000 UHPLC
Mobile phase:	A: water with 0.1% formic acid (FA) B: acetonitrile with 0.1% FA
Gradient:	0-2 min: 10% B; 30min: 60% B; 31min: 95% B for 5 min
Flow rate:	0.3 mL/min
Temperature:	40º C
MS Scan:	The full MS scan was collected at resolution 17,500 with ESI at capillary voltage 3.5 kV in positive ionization mode, S-Lens RF 75, mass range 400 - 5000 Da
Samples:	Humira Innovator (5 mg/mL)* Humira Biosimilar (4 mg/mL)* Yervoy Innovator (5 mg/mL)* Yervoy Biosmilar (3.7 mg/mL)*

**Each sample underwent a 20 h tryptic digest prior to analysis*

Reversed Phase Chromatography

Results

Figure 5: LC/MS analysis of Humira Innovator and Humira Biosimilar Using TSKgel ODS-100V

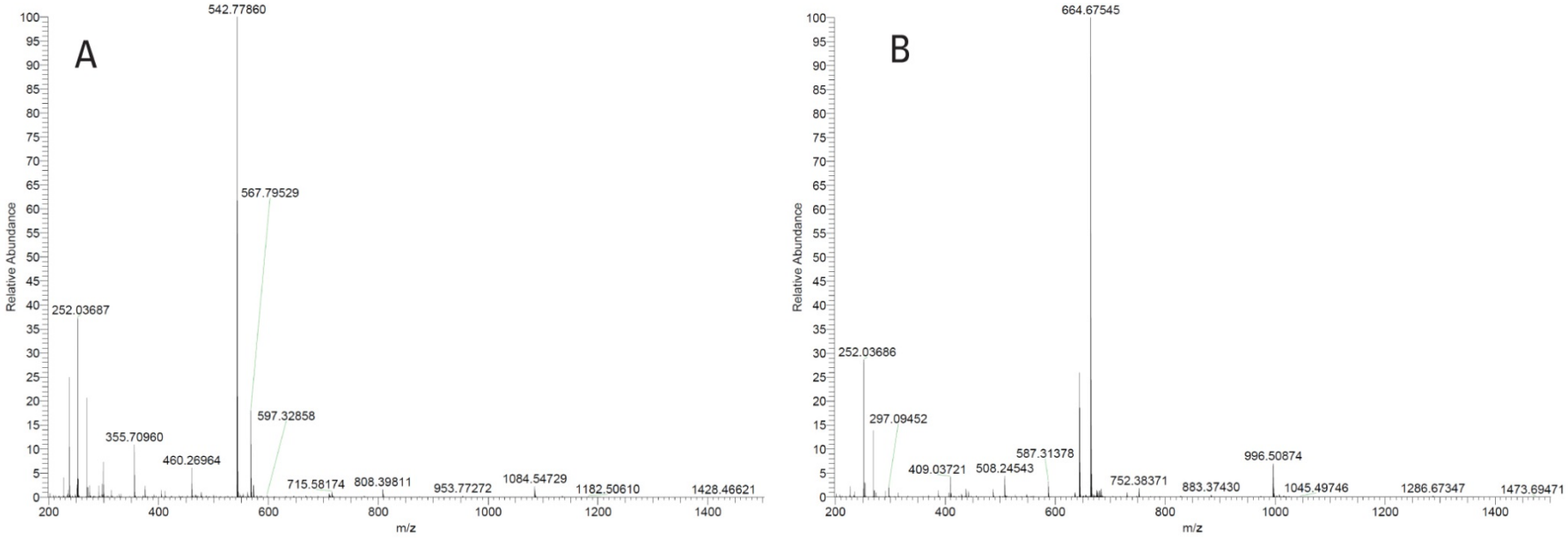


- Biosimilar shows high degree of similarity with the innovator drug.
- Some peaks from biosimilar are not seen in the innovator profile.
- This may be due to variety of reasons; incomplete digestion, some differences in post translational modifications or the absence of some precursors of a few peptide sequences in the innovator molecule[3].

Reversed Phase Chromatography

Results

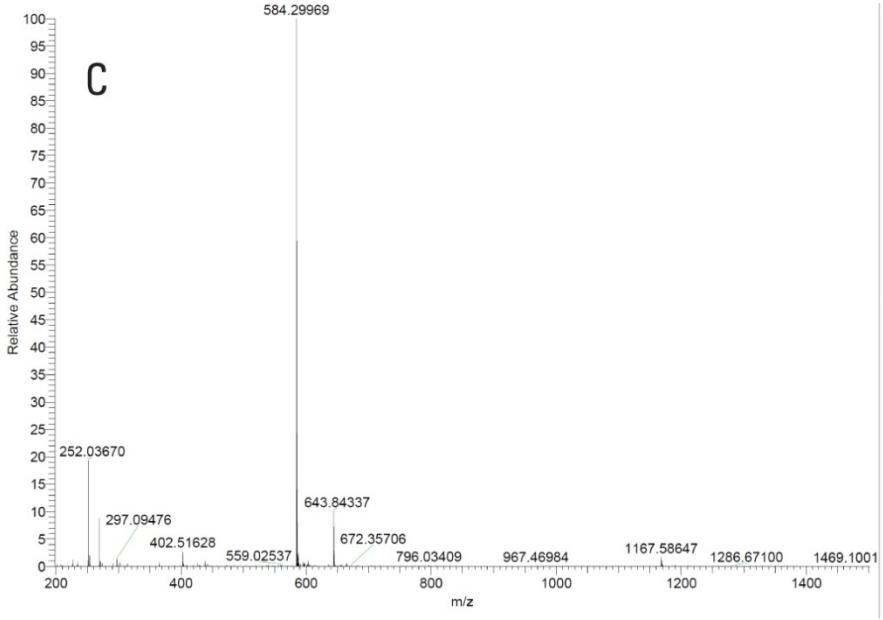
Figure 6: LC/MS Analysis of Novel Peptides Identified in the Total Ion Chromatogram (Figure 5) - A: Peak 1; B: Peak 2

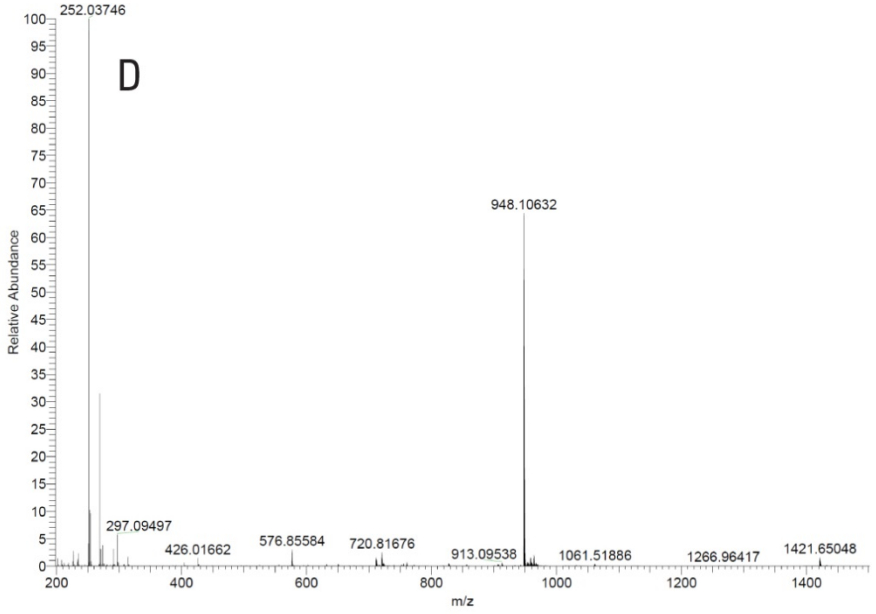


Reversed Phase Chromatography

Results

Figure 7: LC/MS Analysis of Novel Peptides Identified in the Total Ion Chromatogram (Figure 5) - C: Peak 3; D: Peak 4

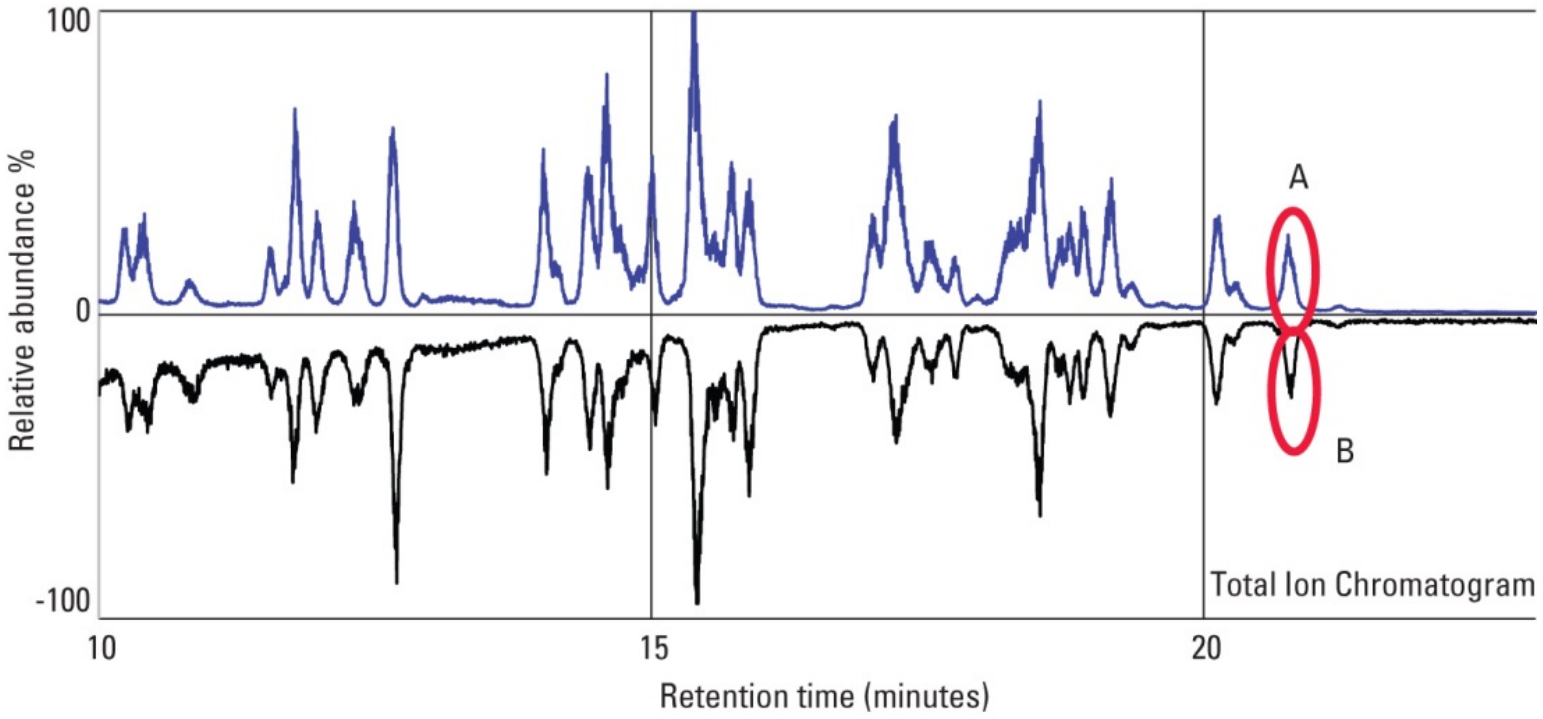




Reversed Phase Chromatography

Results

Figure 8: LC/MS Analysis of Yervoy Innovator and Yervoy Biosimilar Using TSKgel ODS-100V

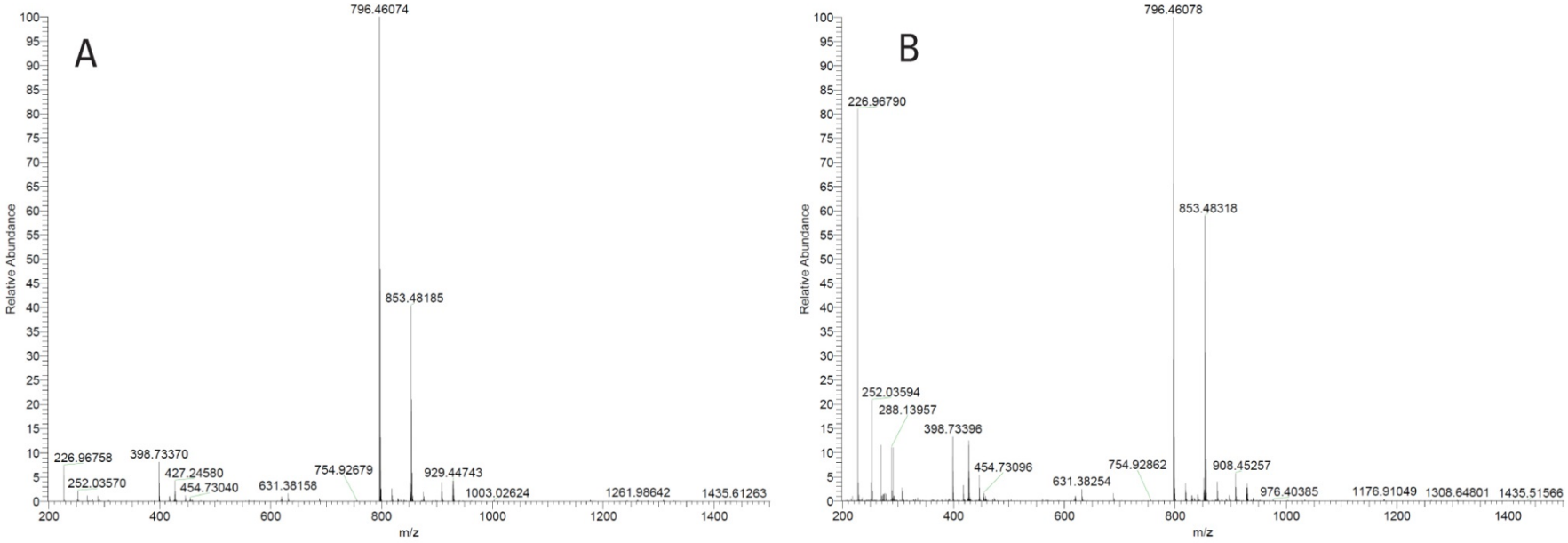


- Biosimilar shows a high degree of similarity compared to the innovator drug.
- Peaks A and B from innovator and biosimilar are used for extraction of mass and comparison, as shown in the next figure.

Reversed Phase Chromatography

Results

Figure 9: LC/MS analysis of “A” and “B” Peptides in the Total Ion Chromatogram (Figure 8)



Hydrophilic Interaction Chromatography (HILIC)

- Hydrophilic interaction liquid chromatography (HILIC) and reversed phase high performance liquid chromatography are complementary techniques in the separation of organic molecules with a broad band of polarity.
- HILIC/MS can therefore expose variations in the presence of peptides that could not be elucidated using RPC/MS when comparing an innovator and a biosimilar.

HILIC Chromatography

Materials and Methods

Column: TSKgel Amide-80, 3 µm, 2.0 mm ID × 15 cm

Table 3: Column Characteristics

Pore size (silica):	8 nm*
Particle size (mean):	3 µm
pH stability:	2.0 - 7.5
Functional group:	carbamoyl
Max. temperature:	50 °C
Surface area:	450 m^2/g

*The pore size of the bonded phase is indicated by the number in the product description, in this case TSKgel Amide-80 has 8 nm nominal pore size. The nominal pore size of the starting base silica is 10 nm.

HILIC Chromatography

Materials and Methods (cont.)

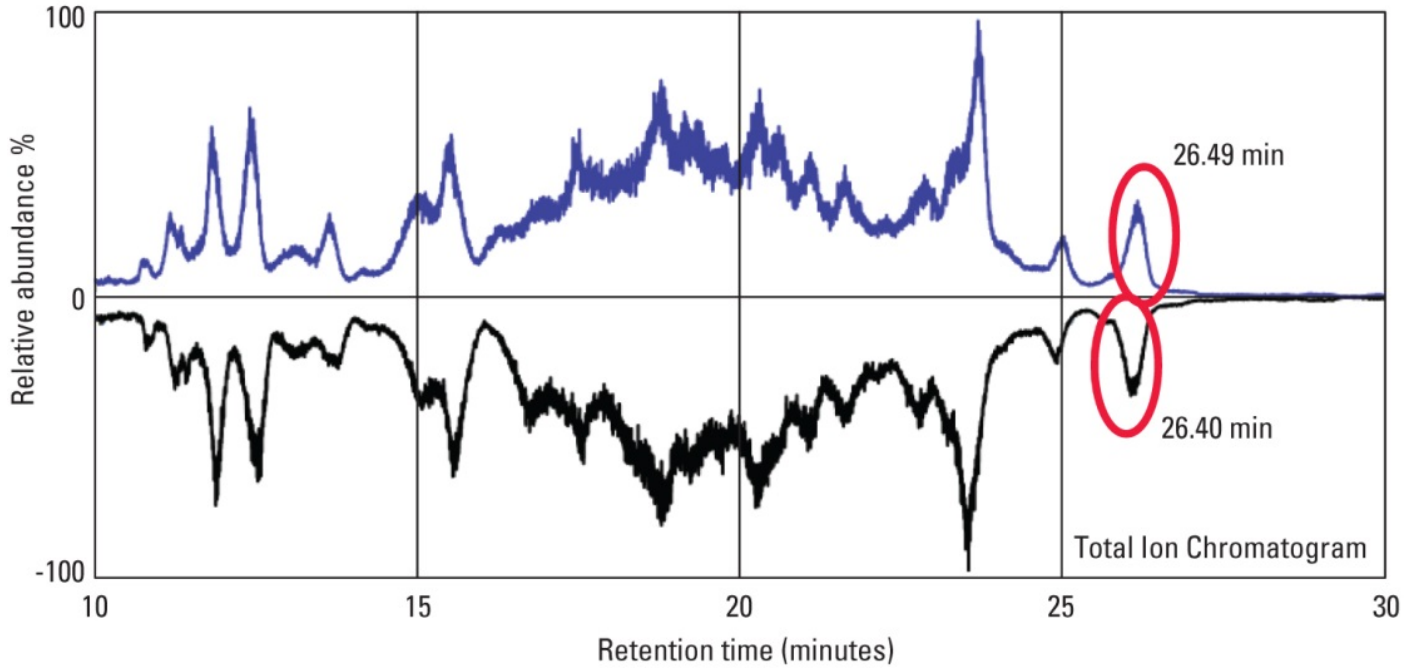
Instrument:	Thermo Orbitrap Q Exactive coupled with Thermo Ultimate 3000 UHPLC
Column:	TSKgel Amide-80, 3 µm, 2.0 mm ID x 15 cm
Mobile phase:	A: water with 0.1% formic acid (FA) B: acetonitrile with 0.1% FA
Gradient:	0-2 min: 90% B; 30min: 50% B; 31min: 0% B for 5 min
Flow rate:	0.3 mL/min
Temperature:	40º C
MS Scan:	The full MS scan was collected at resolution 17,500 with ESI at capillary voltage 3.5 kV in positive ionization mode, S-Lens RF 75, mass range 400 - 5000 Da
Samples:	Yervoy Innovator (5 mg/mL)* Yervoy Biosmilar (3.7 mg/mL)*

**Each sample underwent a 20 h tryptic digest prior to analysis*

HILIC Chromatography

Results

Figure 10: LC/MS Analysis of Yervoy Innovator and Yervoy Biosimilar using TSKgel Amide-80

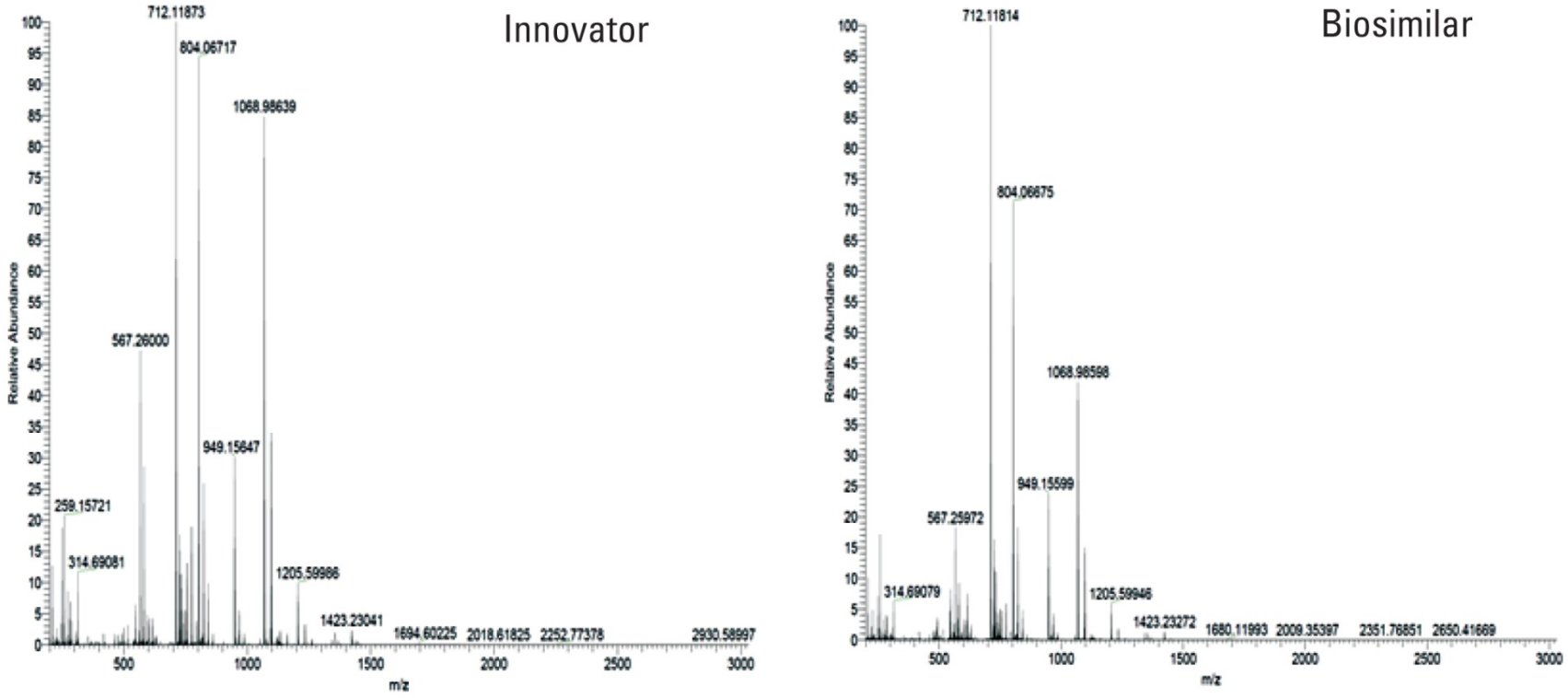


- Optimization of the LC/MS characterization using the HILIC column is still in progress for improvement in sensitivity or ionization.
- Preliminary study shows high similarity between the Yervoy innovator and biosimilar.
- Yervoy peak at RT 26.49 min vs Yervoy biosimilar peak at RT 26.40 min were extracted for mass analysis and shown below.

HILIC Chromatography

Results

Figure 11: LC/MS Analysis of Circled Peptides in the Total Ion Chromatogram (Figure 10)



Hydrophobic Interaction Chromatography (HIC)

Differences in cell line and manufacturing processes can result in minor analytical differences in a proposed biosimilar compared with the innovator, resulting in a slight difference in hydrophobicity. Differences can be elucidated using HIC.

HIC Chromatography

Materials and Methods

Column: TSKgel Butyl-NPR, 5 µm, 4.6 mm ID × 10 cm

Table 3: Column Characteristics

Pore size (mean):	nonporous
Particle size (mean):	2.5 µm
pH stability:	2.0 - 12.0
Functional group:	butyl

HIC Chromatography

Materials and Methods (cont.)

Instrument: Agilent 1100 with Chemstation

Mobile phase: A: 100 mmol/L phosphate buffer, pH 7.0, + 2 mol/L ammonium sulfate
B: 100 mmol/L phosphate buffer

Gradient:

Time	%A	%B
0	100	0
1	50	100
15.1 stop		

Flow rate: 0.5 mL/min

Detection: UV @ 280 nm

Temperature: 25˚ C

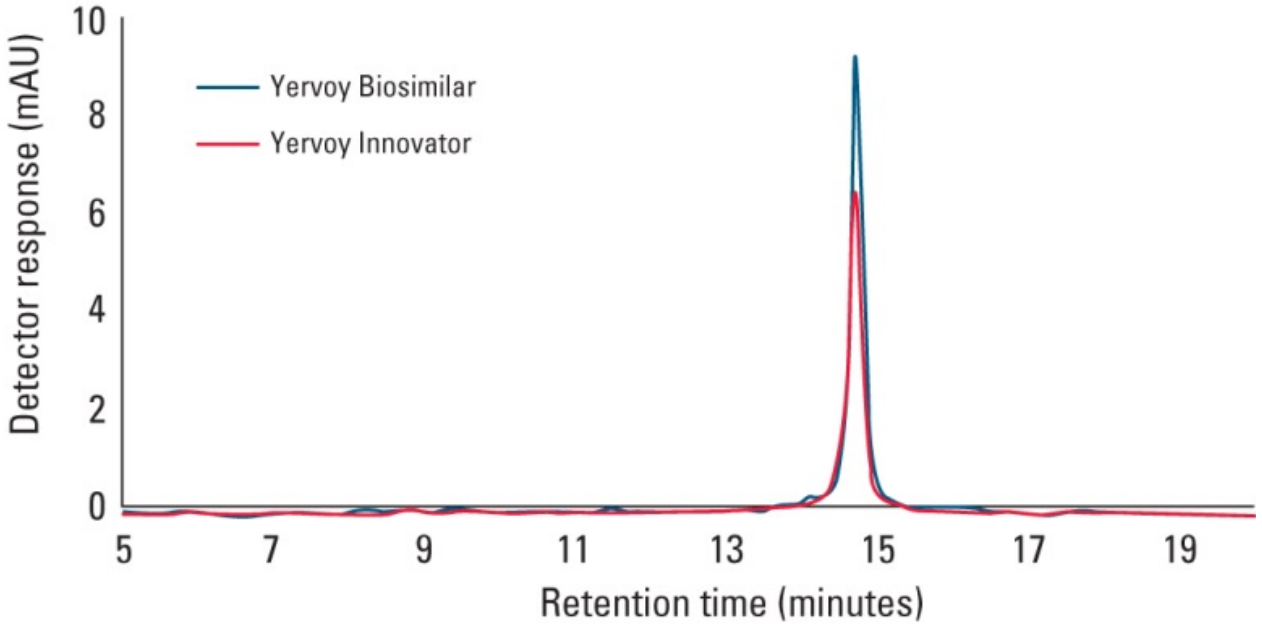
Injection vol.: 5 µL

Samples: Humira Innovator (1 mg/mL)
Humira Biosimilar - Source 1 (1 mg/mL)
Humira Biosimilar - Source 2 (0.09 mg/mL)
Yervoy Innovator (1 mg/mL)
Yervoy Biosimilar (0.8 mg/mL)

HIC Chromatography

Results

Figure 12: HPLC Analysis of Yervoy Innovator and Its Biosimilar Using TSKgel Butyl-NPR

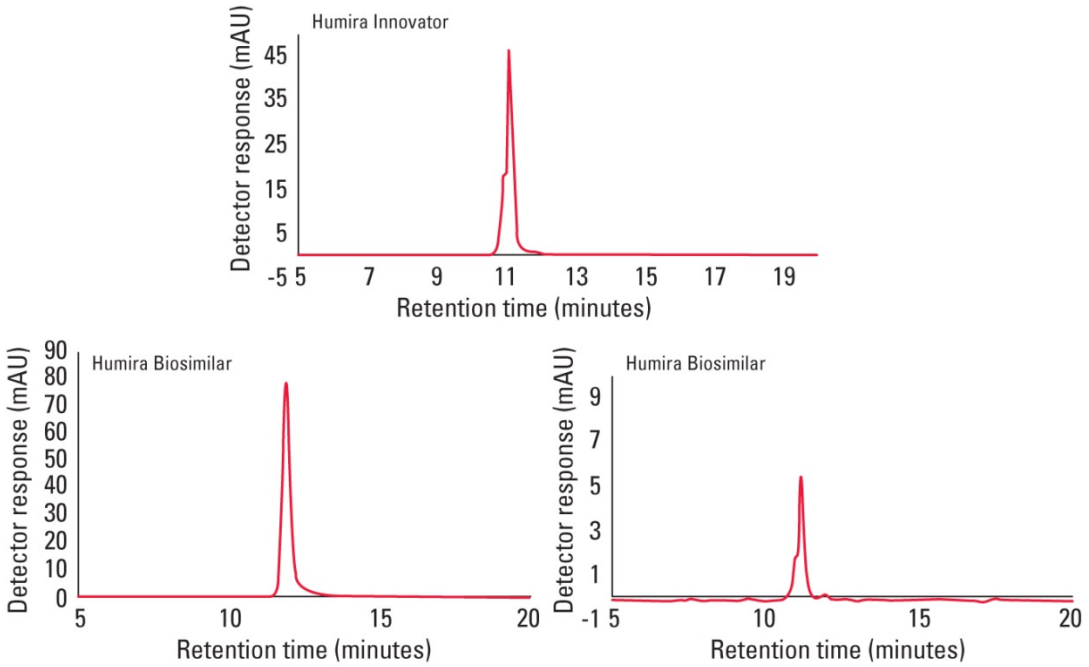


- Both innovator and biosimilar yielded similar separation profile under identical chromatographic conditions - establishing similarity between the two.
- No additional peaks indicating heterogeneity were observed.

HIC Chromatography

Results (cont.)

Figure 13: HPLC Analysis of Humira Innovator and Two Biosimilars Using TSKgel Butyl-NPR



- Humira innovator molecule could be compared to biosimilar molecules from two different sources.
- Slight differences between the biosimilars from two different sources compared to the innovator molecule were observed.

Ion Exchange Chromatography (IEC)

Charge heterogeneity of mAbs can change during the production and purification process. These modifications can potentially impact the stability, efficacy and safety of the drug. Ion exchange chromatography is the method of choice to elucidate charge variants and can be used to unveil similarity between the innovator and biosimilar molecules.

IEC Chromatography

Materials and Methods

Column: TSKgel CM-STAT, 7 µm, 4.6 mm ID × 10 cm

Table 4: Column Characteristics

Particle size (mean):	7 µm and 10 µm
Pore size (mean):	nonporous
Functional group:	carboxymethyl
Counter ion:	NA^+
pH stability:	3.0 - 10.0
Static binding capacity (mg lysozyme/g dry gel):	ca. 20 (7 µm) ca. 15 (10 µm)
Small ion capacity:	100 µeq/g dry gel
pKa:	4.9

IEC Chromatography

Materials and Methods

Column: TSKgel CM-STAT, 7 µm, 4.6 mm ID × 10 cm

Instrument: Agilent 1100 with Chemstation

Mobile phase: A: 10 mmol/L phosphate buffer, pH 7.0
B: 100 mmol/L phosphate buffer + 0.5 mol/L NaCl

Gradient:

Time	%A	%B
0	100	0
25	70	30
30	0	100
35	100	0
35.1 Stop		

Flow rate: 0.5 mL/min

Detection: UV @ 280 nm

Temperature: 25˚ C

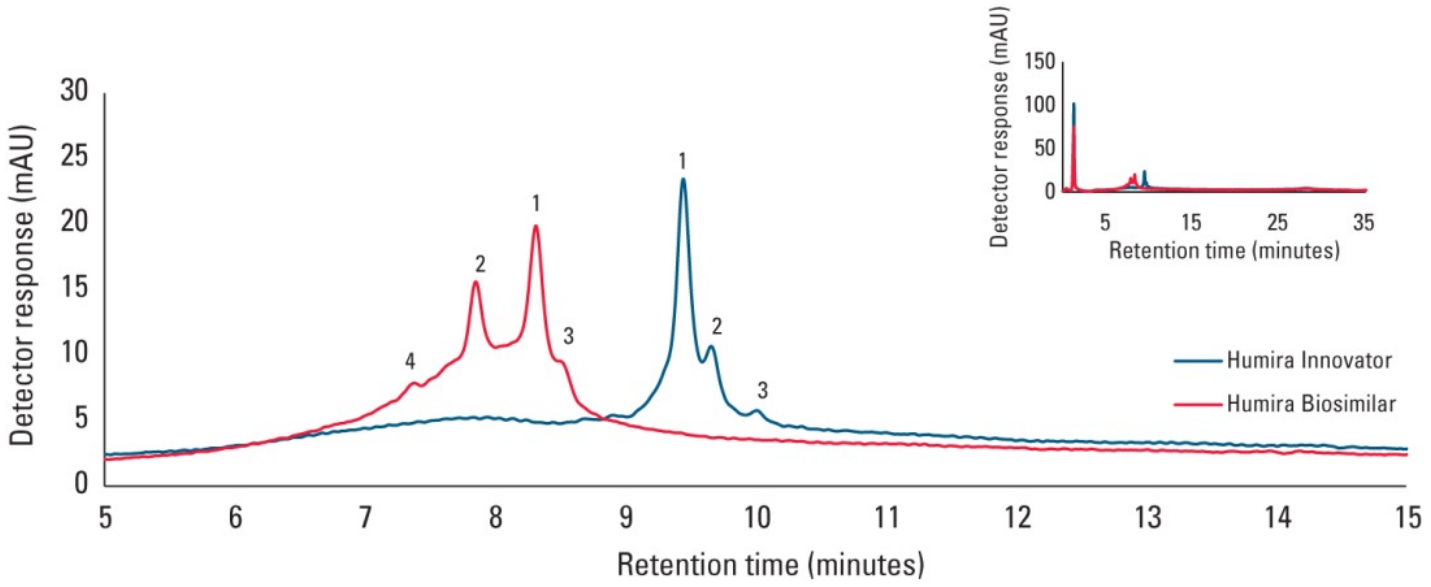
Injection vol.: 5 µL

Samples: Humira® Innovator (1 mg/mL)
Humira Biosimilar (1 mg/mL)
Yervoy Innovator (1 mg/mL)
Yervoy Biosimilar Innovator (1 mg/mL)

IEC Chromatography

Results

Figure 14: Charge Heterogeneity of Humira Innovator and Biosimilar Using TSKgel CM-STAT



This study shows that the Humira innovator and biosimilar have different charge profiles. This variation could indicate a change to the pharmacological properties of the drug.

Summary

This study shows that the following columns from different modes of chromatography can be used for the comparison of an innovator biomolecule with the corresponding biosimilar, as a workflow solution for chromatographic analysis.

Modes	Column	Application
SEC	TSKgel UP-SW3000	Purity analysis; examination of impurities such as aggregate and fragments
RPC	TSKgel ODS-100V	Comparative peptide analysis
HILIC	TSKgel Amide-80	Orthogonal analysis of peptides compared to RPC
HIC	TSKgel Butyl-NPR	Hydrophobic heterogeneity
IEX	TSKgel CM-STAT	Charged isoforms

References

1. FDA: U.S. Food & Drug Administration. (2016). *Demonstration of Comparability of Human Biological Products, Including Therapeutic Biotechnology-derived Products. https://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm122879.htm*

2. Chakrabarti, A. (2018). *Analytical Characterization of a Biosimilar Using a 2 µm Silica Based Size Exclusion Chromatography Column*.

3. MAbs (2011). July-Aug; 2(4): 379-394.