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Middle Down Analysis of HERCEPTIN and its Biosimilar on a Quadrupole-Ion Trap-Orbitrap Mass Spectrometer using Multiple Fragmentations

Xiaoxi Zhang1; Hao Yang2; Haichuan Liu2; Min Du3. 1 ThermoFisher Scientific, Shanghai, China; 2 ThermoFisher Scientific, San Jose, California, US; 3 ThermoFisher Scientific, Massachusetts, US

ABSTRACT

Purpose: To compare HERCEPTIN and its biosimilar on subunit level.

Methods: Multiple fragmentations were combined on a Quadrupole-Ion Trap-Orbitrap mass spectrometer.

Results: The two products are quite similar but still have differences.

INTRODUCTION

A biosimilar is a biologic medical product highly similar to an approved biological medicine (the innovator). Reference to the innovator product is an integral component of the approval. Regulatory authorities such as U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) hold their own guidance on requirements for demonstration of the similarity of two biological products in terms of safety and efficacy.

The monoclonal antibody trastuzumab is marketed by Genentech under the trade name HERCEPTIN® for treatment of breast cancer. HERCEPTIN was approved for medical use in the United States in 1998 and a biosimilar was approved in the United States in 2018. In this study, we evaluated similarity of HERCEPTIN and a biosimilar candidate by middle-down analysis using multiple fragmentation methods. Middle down is faster and peptide mapping provides more details. The two methods complement and verify each other.

MATERIALS AND METHODS

Sample Preparation

The samples, which were provided by a collaborator, were digested with IdeS protease (Promega) following the protocol provided by the company.

Liquid Chromatography

5μg of the digested samples were separated on a Thermo Scientific™ Vanquish™ HPLC System (A: 0.1% formic acid in water, B: 0.1% formic acid in acetonitrile) coupled with a Thermo Scientific™ MAbPac™ RP (2.1 mm × 50 mm, 4 μm) column. The separation gradient shows in Table 1.

Mass Spectrometry

A Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ Quadrupole-Ion Trap-Orbitrap Mass Spectrometer was used for all data collection.

Data Analysis

Data analysis was performed using Thermo Scientific™ BioPharma Finder™ Software(v3.2).

Table 1. Chromatography gradient

Time(min)	%B
0.0	20
1.0	20
8.0	35
9.0	80
9.5	80
10	20
12	20

Table 2. Middle down instrument method

Middle Down Instrument Method	
Spray Voltage: Positive Ion (V)	3600
Ion Transfer Tube Temp (℃)	275
Vaporizer Temp (℃)	200
Resolution	120,000
Fragmentation	HCD 8%; ETD 8ms; UVPD 20ms
Isolation Window (Da)	1.6
m/z range (Da)	350-2000
Max. injection time (ms)	250
Microscan	5
Maximum number of multiplexed ions	5

RESULTS

Intact mass analysis of subunits

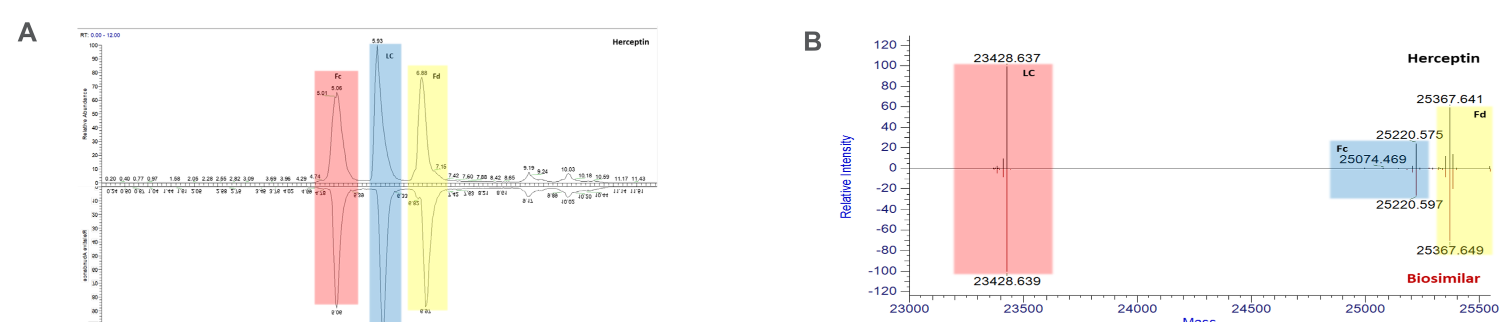
HERCEPTIN and biosimilar samples were digested with IdeS, and the three subunits (LC, Fc, and Fd) were baseline separated and detected (Figure 1).

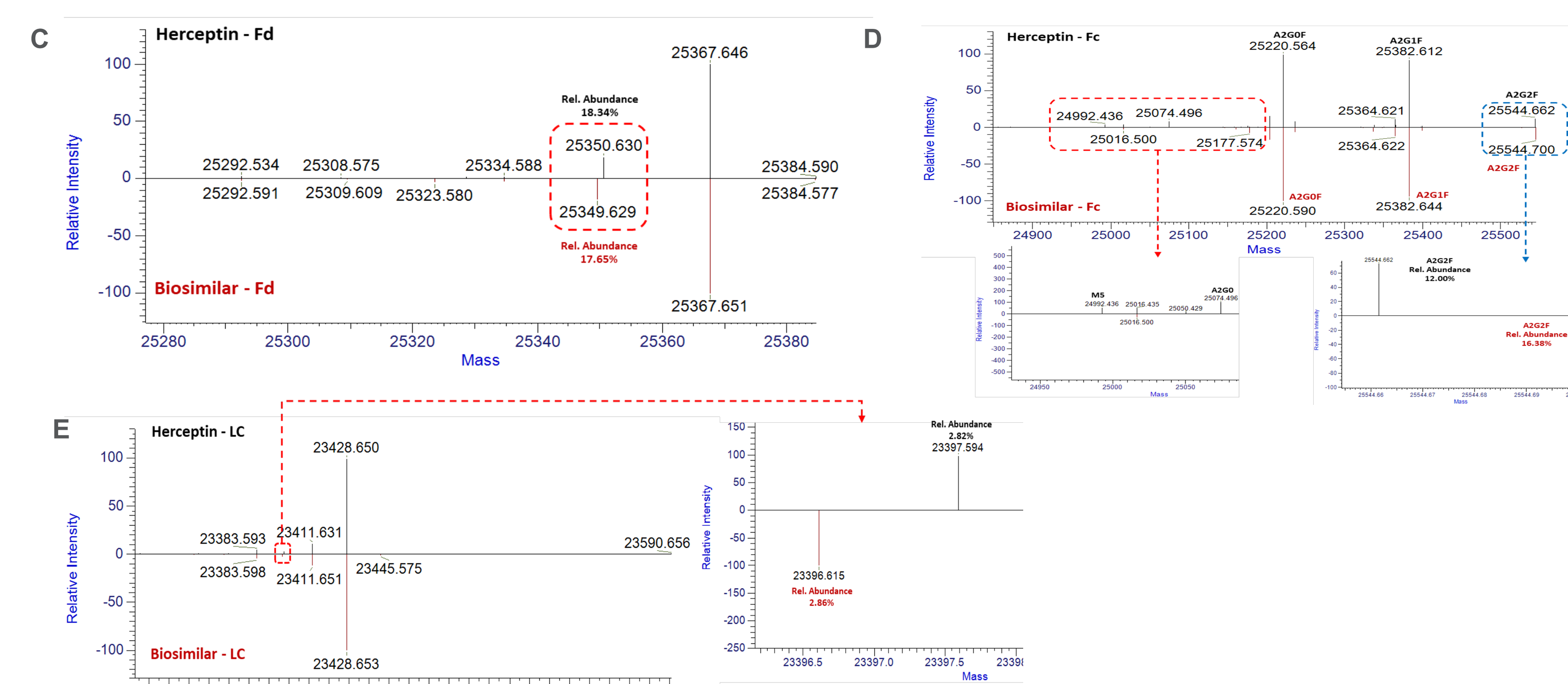
Subunit level comparison

Figure 1 show the subunit level comparison of HERCEPTIN innovator and biosimilar.

It was found that the two products are quite similar but still have differences. For example, in the Fd region, the second most abundant peak of the innovator was found at MW 25,350.630 (18.34%) while MW 25,349.629 (17.65%) was the second most abundant peak for the biosimilar (Figure 1C).

Figure 1. Subunit level comparison of HERCEPTIN innovator and biosimilar. A, chromatography mirror plot. B, deconvolution mirror plot overview. C, Fd region. D, Fc region. E, LC region. Differences are all labelled on the figures.





In the Fc region the relative abundance of A2G2F is 12.00 % in the innovator as compared to 16.38 % in the biosimilar. The glycoforms such as A2G0 and M5 were identified in the innovator only (Figure 1D). In the light chain, a peak with MW of 23,397.594 (2.82%) was detected in the innovator while 23,396.615 (2.86%) was found in the biosimilar (Figure 1E).

Middle down method optimization

For each subunit, 5 different charge state target ions were selected for middle down fragmentation. Figure 2B shows the top abundance ion of each subunit as an example. The complete list shows in table 3.

Then multiple MS/MS fragmentations, including ETD, UVPD and HCD, were used to obtain sequence information of the targeted subunits. The reaction time/energy were optimized for getting high-quality MS/MS spectra: HCD (8%), ETD (8ms) and UVPD (20ms). The optimized method shows in table 2.

After optimization, for both products, the middle down sequence coverage was >80% for Fc region and LC (data not shown) and >75% for Fd region (Figure 3).

Figure 2. Subunit mass spectra and middle down target ions. A, mass spectra across full mass range. B, target ions.

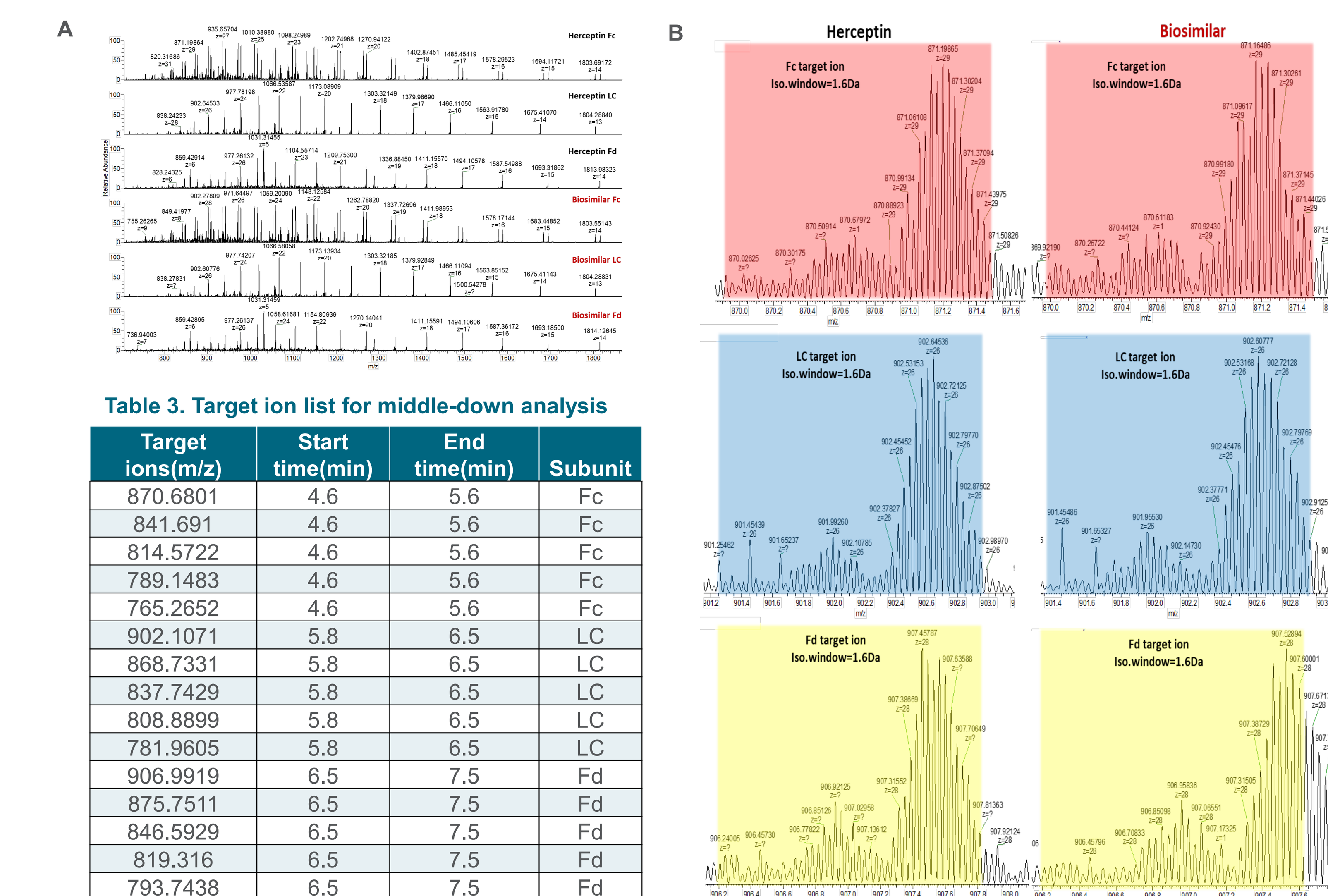


Table 3. Target ion list for middle-down analysis

Target ions(m/z)	Start time(min)	End time(min)	Subunit
870.6801	4.6	5.6	Fc
841.691	4.6	5.6	Fc
814.5722	4.6	5.6	Fc
789.1483	4.6	5.6	Fc
765.2652	4.6	5.6	Fc
902.1071	5.8	6.5	LC
868.7331	5.8	6.5	LC
837.7429	5.8	6.5	LC
808.8899	5.8	6.5	LC
781.9605	5.8	6.5	LC
906.9919	6.5	7.5	Fd
875.7511	6.5	7.5	Fd
846.5929	6.5	7.5	Fd
819.316	6.5	7.5	Fd
793.7438	6.5	7.5	Fd

Modification site determination of Fd region

We've got high sequence coverage for all three subunits, and the 1Da difference in Fd region especially attracted our attention. To determine where is difference came from, we focused on the middle down results of Fd region. By comparing Fd region middle down sequence maps of these two products, two suspicious areas were found and highlighted(Figure 3).

With these two candidates, we move further to check the fragment ions, try to make sure where exactly the modification happened. The top down search results generated by BiopharmaFinder could provide us a lot of details, such as sequence map, spectra comparison, annotated fragment ions spectrum and matching fragment detail (Figure 4).

Figure 4A showed the Fd region MS spectra comparison of the two products. It is clearly that on MS level, mass difference could be observed. The innovator shifted a little to high mass range.

Figure 4B is the matching fragment detail table of HERCEPTIN of one suspicious area, "HWVR". Following it we checked the A36(3+)ions generated by UVPD and 1Da difference was found in fragment ions comparison(Figure 4C). So we could lock the modification site should be W36 on heavy chain (Figure 4D, highlighted).

Figure 3. Middle down sequence map of HERCEPTIN and its biosimilar on subunit level, takes Fd region as example.

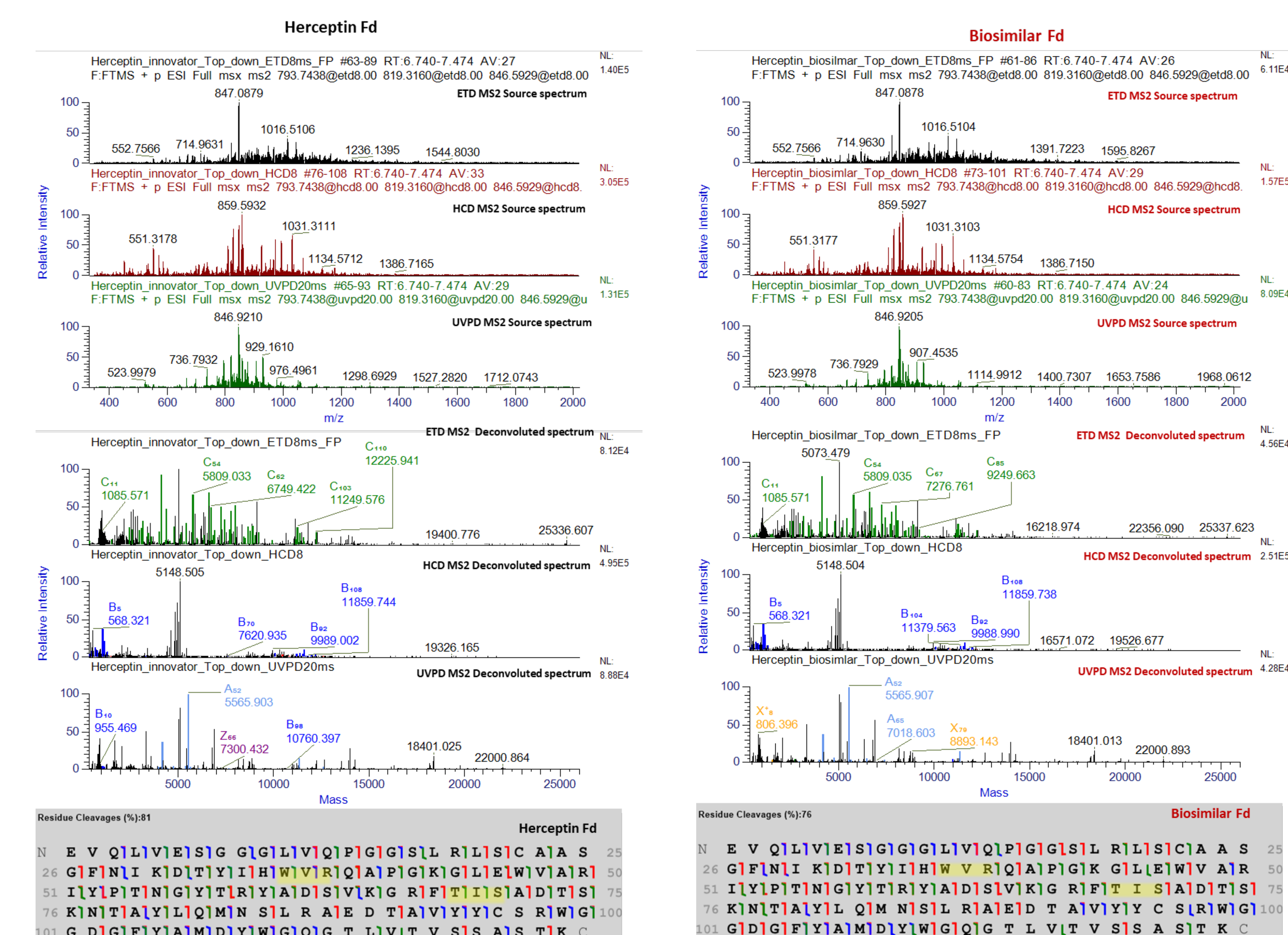
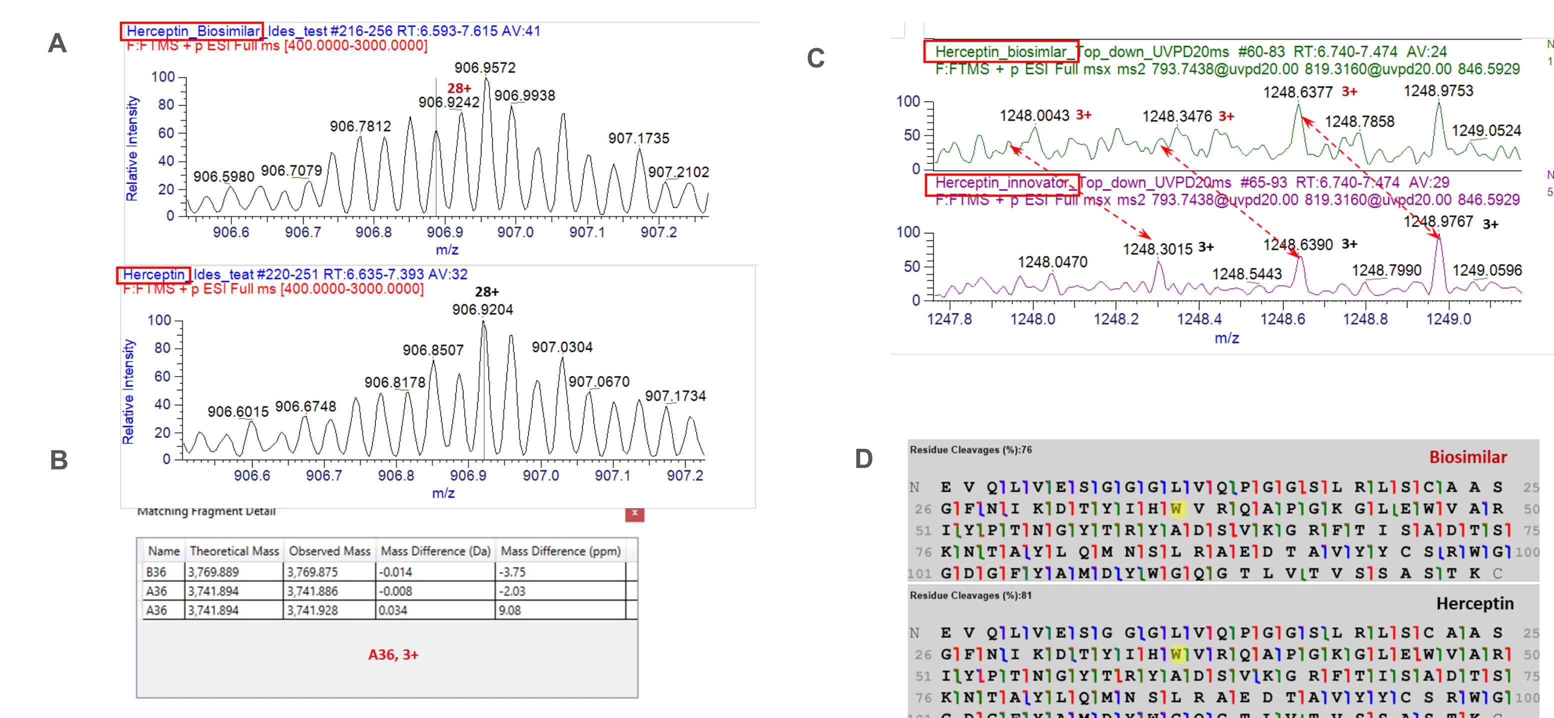


Figure 4. The spectra of modification site on Fd region. A, MS spectra comparison. B, matching fragment details of HERCEPTIN. C, UVPD spectra comparison. D, sequence coverage map.



CONCLUSIONS

- Some differences with subunit mass was observed between HERCEPTIN and it's biosimilar, which lead to further investigation using middle down approach.
- Using a multiple fragmentations combined middle down approach, we obtained information rich, high quality fragmentation data.
- We achieved high sequence coverage and successfully pinpointed the modification site which caused 1 Da mass difference in Fd region between innovator and biosimilar.
- Middle down is a faster characterization technology compare to peptide mapping to meet the needs for quicker turn around time and decision making, such as during bioprocessing development and monitoring. It can serve as a complementary method to peptide mapping.

TRADEMARKS/LICENSING

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