



# Identifying Monoclonal Antibody Mutation Sites Using the Agilent 1290 Infinity II 2D-LC Solution with Q-TOF LC/MS

## Application Note

Biopharmaceuticals and Biosimilars

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### Abstract

In recent years, 2D-LC has been highly promising for the detailed characterization and comparability assessment of protein biopharmaceuticals such as monoclonal antibodies. This Application Note describes the use of the Agilent 1290 Infinity II 2D-LC solution in the comparison of an infliximab originator and candidate biosimilar. RPLC×RPLC and SCX×RPLC peptide mapping revealed important differences that could be attributed to a double mutation in the heavy chain of the candidate biosimilar using quadrupole time-of-flight mass spectrometry.



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## Introduction

Monoclonal antibodies (mAbs) have emerged as important biopharmaceuticals. Today, more than 40 mAbs are marketed in the United States and Europe, of which 18 have attained blockbuster status, with sales more than doubled since 2008<sup>1,2,3</sup>. Over 50 are in late-stage clinical development.

The knowledge that the top-selling mAbs are, or will become, open to the market in the coming years has resulted in an explosion of biosimilar activities. In 2013, we witnessed the European approval of the first two mAb biosimilars (Remsima and Inflectra), which both contain the same active substance, infliximab<sup>4</sup>. In April 2016, Inflectra also reached marketing authorization in the US, and a third infliximab biosimilar (Flixabi) was recently approved in Europe. Remicade, infliximab's originator, reached global sales of \$8.9 billion in 2013<sup>3</sup>.

It is clear that the biosimilar market holds great potential, but it is simultaneously confronted with major hurdles. In contrast to generic versions of small molecules, exact copies of recombinant mAbs cannot be produced due to differences in the cell clone and manufacturing processes used. Even originator companies experience lot-to-lot variability. As a consequence, regulatory agencies evaluate biosimilars based on their level of similarity to, rather than the exact replication of, the originator. In demonstrating similarity, an enormous weight is placed on analytics, and both biosimilars and originators need to be characterized and compared in great detail. In contrast to small-molecule drugs, mAbs are large (approximately 150 kDa) and heterogeneous (due to the biosynthetic process and subsequent manufacturing and storage), making their analysis highly challenging<sup>1,2</sup>.

Online two-dimensional liquid chromatography (2D-LC) is an emerging tool used to tackle this analytical complexity<sup>5</sup>. In online 2D-LC, peaks, parts, or the whole chromatogram are subjected to two different separation mechanisms. Online 2D-LC can be divided into two main types. In comprehensive two-dimensional LC (LC×LC), the entire effluent stream of the first (<sup>1</sup>D) column is transferred to the second (<sup>2</sup>D) column. In heart-cutting two-dimensional LC (LC-LC), one peak or one part of the chromatogram is transferred to the <sup>2</sup>D column. Multiple peaks or multiple parts of the chromatogram also can be selected for transfer to the <sup>2</sup>D column (mLC-LC). Multiple heart-cutting two-dimensional LC (mCEX-RPLC) combined with high-resolution MS has recently been used at the protein level to identify the main isoforms of the mAb rituximab, and to characterize the antibody drug conjugate (ADC) ado-trastuzumab emtansine (marketed as Kadcyra)<sup>6,7</sup>. Comprehensive LC×LC has shown promise at the peptide level for the detailed characterization and comparability assessment of a Herceptin innovator and biosimilar, and assessment of drug conjugation sites in Kadcyra<sup>7,8,9,10</sup>.

To further illustrate the attractiveness of LC×LC for the detailed characterization and comparability assessment of biopharmaceuticals, we describe the comparison of the tryptic digests of the originator infliximab (Remicade) and a biosimilar candidate for which the recombinant expression in Chinese hamster ovary (CHO) cells was going wrong. An Agilent 1290 Infinity II 2D-LC solution, operated in LC×LC mode, was combined with an Agilent 6530 Quadrupole Time-of-Flight (Q-TOF) LC/MS.

## Experimental

### Materials

Acetonitrile, methanol, formic acid (FA), and water were acquired from Biosolve (Valkenswaard, The Netherlands). Trifluoroacetic acid, dithiothreitol (DTT), iodoacetamide (IAM), NaCl, NH<sub>4</sub>HCO<sub>3</sub>, NaH<sub>2</sub>PO<sub>4</sub>, and phosphoric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA), and porcine sequencing-grade modified trypsin was from Promega (Madison, MA, USA). Tris-HCl was from Thermo Fisher Scientific (Waltham, MA, USA), and Rapigest was from Waters (Milford, MA, USA). Monoclonal antibodies were obtained from a local biotechnology company.

### Sample preparation

#### Preparation of light and heavy chains

A 100 mM amount of DTT was added to the sample to a final concentration of 10 mM DTT following sample dilution to 0.2 mg/mL using 100 mM Tris-HCl, pH 8.0. Reduction was performed at 60 °C for 1 hour.

#### Trypsin digestion

To a volume corresponding to 100 µg of protein, 105 µL of 0.1 % Rapigest in 100 mM Tris-HCl, pH 8, was added followed by the addition of 100 mM Tris-HCl, pH 8, to a final volume of 192.5 µL. The sample was subsequently reduced at 60 °C for 30 minutes by the addition of 5 mM DTT (2.5 µL of 400 mM DTT in 100 mM Tris-HCl), and alkylated at 37 °C for 1 hour by adding 10 mM IAA (5 µL of 400 mM IAA in 100 mM Tris-HCl). Digestion proceeded for 16 hours at 37 °C using trypsin as protease, added at an enzyme-to-substrate ratio of 1:25 (w:w). Lyophilized trypsin (20 µg) dissolved in 100 mM Tris-HCl (50 µL) was added in a volume of 10 µL, for a final sample volume of 210 µL.

### LC instrumentation (middle-up)

- Agilent 1290 Infinity II High Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity II Diode Array Detector (G7117B) equipped with a 10-mm flow cell
- Agilent 6540 Accurate-Mass Quadrupole Time-of-Flight LC/MS (G6540A)

### 2D-LC instrumentation (peptide mapping)

- Agilent 1290 Infinity II High Speed Pump (G7120A) with seal wash option (first dimension)
- Agilent 1290 Infinity II High Speed Pump (G7120A) (second dimension)
- Agilent 1290 Infinity II Multisampler (G7167B)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity Valve Drive (G1170A)
- Agilent InfinityLab Quick Change 2-position/4-port duo valve for 2D-LC equipped with two 40- $\mu$ L loops (G4236A)
- Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight LC/MS (G6530A)

### Software

- Agilent OpenLab CDS ChemStation, revision C.01.07 with 2D-LC add-on software, revision A.01.02
- Agilent Technologies MassHunter for instrument control (B05.01)
- Agilent Technologies MassHunter for data analysis (B07.00)
- Agilent Technologies BioConfirm software for MassHunter (B07.00)
- GC Image LC $\times$ LC Edition Software for 2D-LC data analysis (GC Image, LLC., Lincoln, NE, USA)

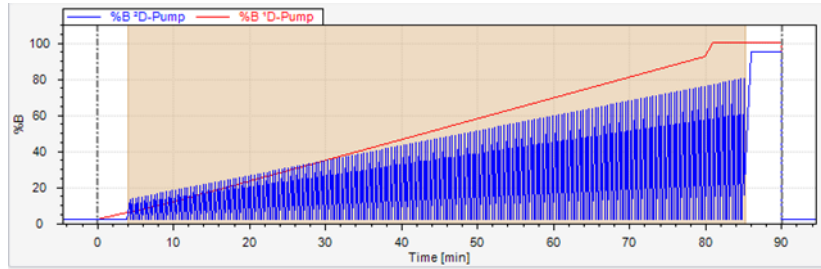
### LC/MS method parameters (middle-up analysis)

RPLC-UV-MS	
Column	Agilent AdvanceBio RP-mAb C4, 50 $\times$ 4.6 mm, 3.5 $\mu$ m
Solvent A	0.1 % TFA
Solvent B	0.1 % TFA in ACN
Flow rate	1 mL/min
Gradient	0 to 6 minutes – 30 to 42.5 %B 6 to 6.1 minutes – 42.5 to 95 %B 6.1 to 7.1 minutes – 95 %B 7.1 to 7.2 minutes – 95 to 30 %B 7.2 to 9.2 minutes – 30 %B
Temperature	80 $^{\circ}$ C
Injection	
Volume	10 $\mu$ L
Temperature	4 $^{\circ}$ C
Needle wash	6 seconds flush port (solvent B)
Detection DAD	
Wavelength	Signal 214/8 nm
Detection MS	
Ionization	Agilent Jet Stream technology source, positive ionization
Drying gas	350 $^{\circ}$ C, 10 L/min
Nebulizer	50 psig
Sheath gas	350 $^{\circ}$ C, 11 L/min
Capillary voltage	3,500 V
Nozzle voltage	1,000 V
Fragmentor	200 V
Acquisition	High resolution (4 GHz), resolution 40,000 for $m/z$ 1,000, 2 spectra/s

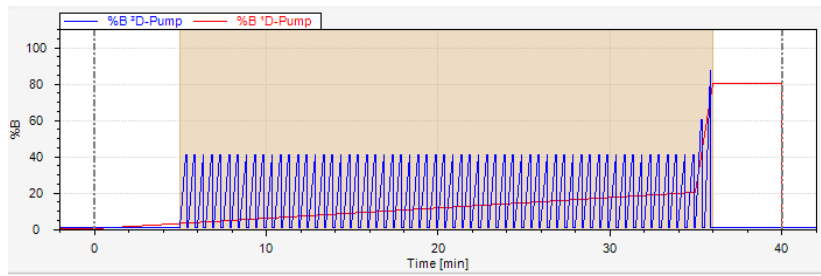
## LC×LC/MS method parameters (peptide mapping)

	SCX×RPLC	RPLC×RPLC
<b>First dimension</b>		
Column	Agilent Bio SCX, NP 10 (PEEK), 2.1 × 250 mm, 10 μm	Agilent ZORBAX Bonus-RP, 2.1 × 150 mm, 3.5 μm
Solvent A	10 mM Phosphate pH 3	10 mM NH <sub>4</sub> -Bicarbonate pH 8.2
Solvent B	10 mM Phosphate pH 3 + 1 M NaCl	MeOH:ACN 50:50
Flow rate	65 μL/min	80 μL/min
Gradient	0 to 35 minutes – 0 to 20 %B 35 to 36 minutes – 20 to 80 %B 36 to 40 minutes – 80 %B Post time – 10 minutes at 0 %B	0 to 80 minutes – 2 to 92 %B 80 to 81 minutes – 92 to 100 %B 81 to 90 minutes – 100 %B Post time – 9 minutes at 2 %B
Temperature	23 °C	23 °C
<b>Second dimension</b>		
Column	Agilent ZORBAX Eclipse Plus C18, 4.6 × 50 mm, 3.5 μm	Agilent ZORBAX Eclipse Plus C18, 4.6 × 50 mm, 3.5 μm
Solvent A	0.1 % Formic acid in water	0.1 % Formic acid in water
Solvent B	ACN	ACN
Flow rate	3.5 mL/min	3.5 mL/min
Idle Flow rate	0.4 mL/min	0.5 mL/min
Initial gradient	0 to 0.35 minutes – 1 to 41 %B 0.35 to 0.40 minutes – 41 %B 0.40 minutes – 1 %B	0 to 0.35 minutes – 2 to 10 %B 0.35 minutes – 2 %B
Gradient modulation	Full-in-fraction with increased %B at end for cleaning 1 %B at 0 minutes 41 %B at 0.35 minutes to 41 %B at 35 minutes to 95 %B at 36 minutes	Constantly shifted %B and Δ%B 2 %B at 0 minutes to 2 %B at 85 minutes to 95 %B at 86 minutes (10 %B at 0.35 minutes to 80 %B at 85 minutes)
Temperature	50 °C	55 °C
<b>Modulation</b>		
Modulation on	5 to 36 minutes	4 to 85 minutes
Loops	Two 40-μL loops, cocurrent configuration	Two 40-μL loops, cocurrent configuration
Modulation time	0.50 minutes	0.40 minutes
<b>Injection</b>		
Volume	8 μL	8 μL
Temperature	4 °C	4 °C
Needle wash	6 seconds flush port (50 % ACN)	6 seconds flush port (50 % ACN)
<b>Detection MS</b>		
Ionization	Agilent Jet Stream technology source, positive ionization	Agilent Jet Stream technology source, positive ionization
Drying gas	350 °C, 11 L/min	350 °C, 11 L/min
Nebulizer	55 psig	55 psig
Sheath gas	400 °C, 9 L/min	400 °C, 9 L/min
Capillary voltage	3,500 V	3,500 V
Nozzle voltage	1,000 V	1,000 V
Fragmentor	175 V	175 V
<b>Acquisition</b>		
	Extended Dynamic Range (2 GHz)	Extended Dynamic Range (2 GHz)
Resolution	10,000 for <i>m/z</i> 1,000	10,000 for <i>m/z</i> 1,000
MS	8 spectra/s	8 spectra/s
MS/MS	Data Dependent MS/MS, 8 spectra/s	Data Dependent MS/MS, 8 spectra/s
MS/MS Threshold (Abs)	1,000	1,000
Isolation width	~4 <i>m/z</i>	~4 <i>m/z</i>
Collision energy	(3.6 × <i>m/z</i> )/100–4	(3.6 × <i>m/z</i> )/100–4

### RPLC×RPLC gradient



### SCX×RPLC gradient



## Results and Discussion

Figure 1A shows the LC/UV-MS middle-up analysis of an infliximab originator and a candidate biosimilar after chemical reduction to cleave the disulfide bridges. Separation was obtained on a wide-pore superficially porous C4 column (AdvanceBio RP-mAb). The light-chain (Lc) peaks nicely overlay, but a retention difference is noted for the heavy-chain (Hc) peaks. When combining the separation with Q-TOF MS, a 99-Da mass increase is demonstrated on the biosimilar Hc ( $m/z$  50,833.0 in the originator, and 50,931.9 in the biosimilar) explaining this retention time shift (Figure 1B).

LC×LC peptide mapping was performed subsequently to elucidate the origin of this retention time and mass difference. Compared to 1D-LC, LC×LC is known to increase substantially the chromatographic resolution as long as the two dimensions are orthogonal, and the separation obtained in the first dimension is maintained upon transfer to the second dimension. Orthogonal combinations for 2D-LC-based peptide mapping are: strong cation exchange × reversed-phase LC (SCX×RPLC), hydrophilic interaction chromatography × reversed-phase LC (HILIC×RPLC) and reversed-phase LC × reversed-phase LC (RPLC×RPLC) at different pH values in the two dimensions<sup>8</sup>.

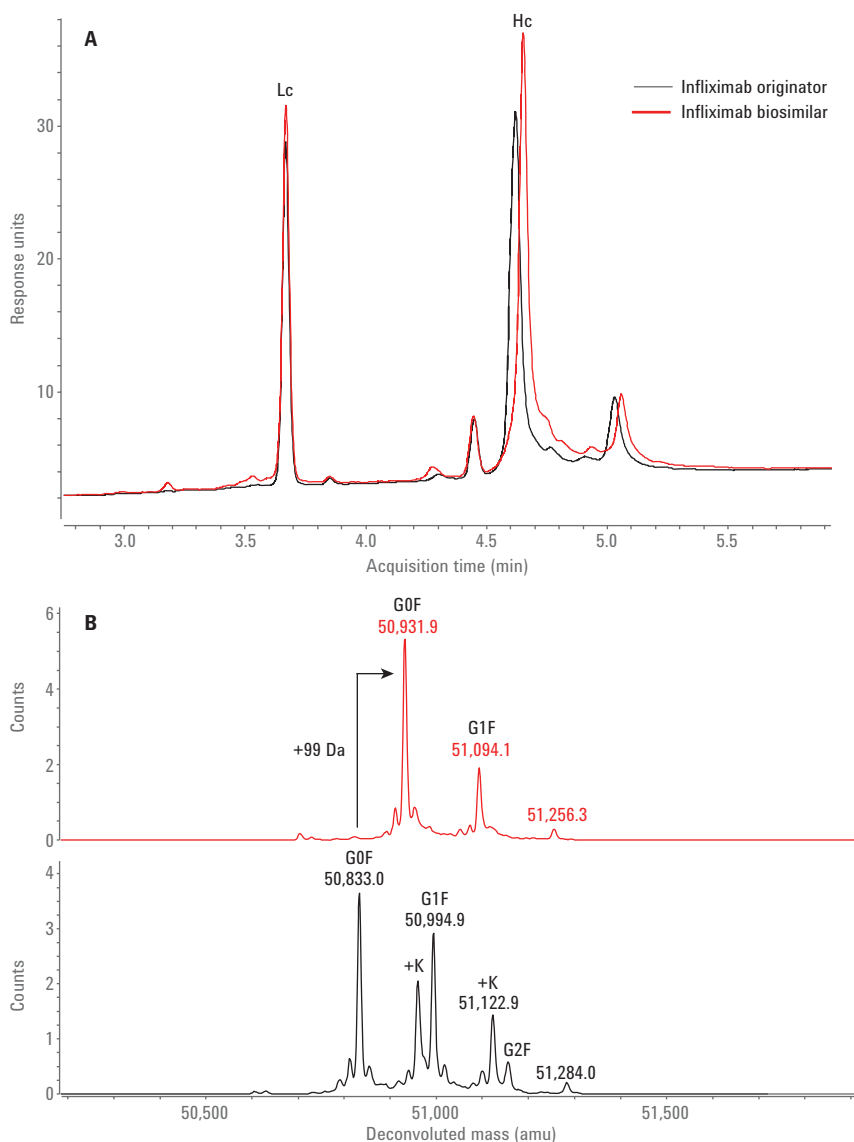


Figure 1. RPLC/Q-TOF MS middle-up analyses of infliximab originator (black) and candidate biosimilar (red) on an Advance Bio RP-mAb column. A) Chromatogram showing the Lc and Hc peaks, and (B) the deconvoluted mass spectra recorded for the Hc showing the glycoforms (GOF, G1F, and G2F) and the +99-Da shift.

Figure 2 displays the LC×LC tryptic peptide maps of the infliximab originator and candidate biosimilar using the combination RPLC×RPLC. Both plots are similar, but some striking differences are noted, taking the MS data into consideration. The spots SLSLSPG and SLSLSPGK clearly present in the originator are replaced by one spot SLSLSPGI in the biosimilar, which, according to the mass spectral data, corresponds to the addition of an isoleucine (I) to SLSLSPG, or the replacement of lysine (K) by isoleucine in SLSLSPGK at the C-terminus of the heavy chain. From a biochemical standpoint, this makes sense and results in a positive move of 113 Da. The origin of the two spots SLSLSPG and SLSLSPGK in the originator mAb can be explained by the knowledge that heavy chains are historically cloned with a C-terminal lysine, but during cell culture production, host cell carboxypeptidases act on the antibody, resulting in the partial removal of these lysine residues. The presence of the lysine residues is also apparent in the deconvoluted Q-TOF MS spectra shown in Figure 1B. A small retention shift was also noted in another spot that could be identified by MS/MS as NYYGSTYDYWGQGTTLVSSASTK in the originator, and as NYYGSSYDYWGQGTTLVSSASTK in the biosimilar (Figure 3). From threonine (T) to serine (S) is -14 Da, and combining both modifications (+113 Da, -14 Da) results in a difference of 99 Da. Two point mutations are at the origin of this wrong recombinant expression (Figure 2). According to US and European regulatory authorities, identical primary sequence is primordial to similarity, thereby ruling out this candidate biosimilar from further development.

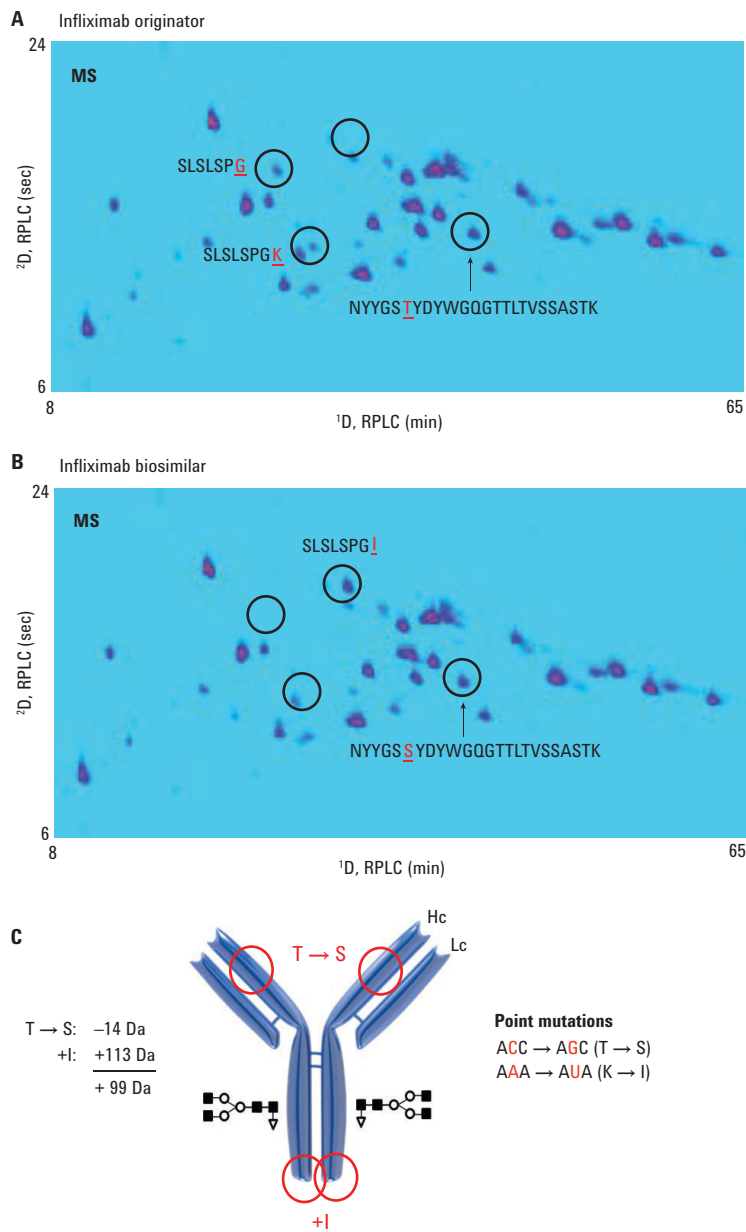


Figure 2. RPLC×RPLC/Q-TOF MS analysis of infliximab originator and candidate biosimilar, and schematic of the mAb with annotation of the modifications.

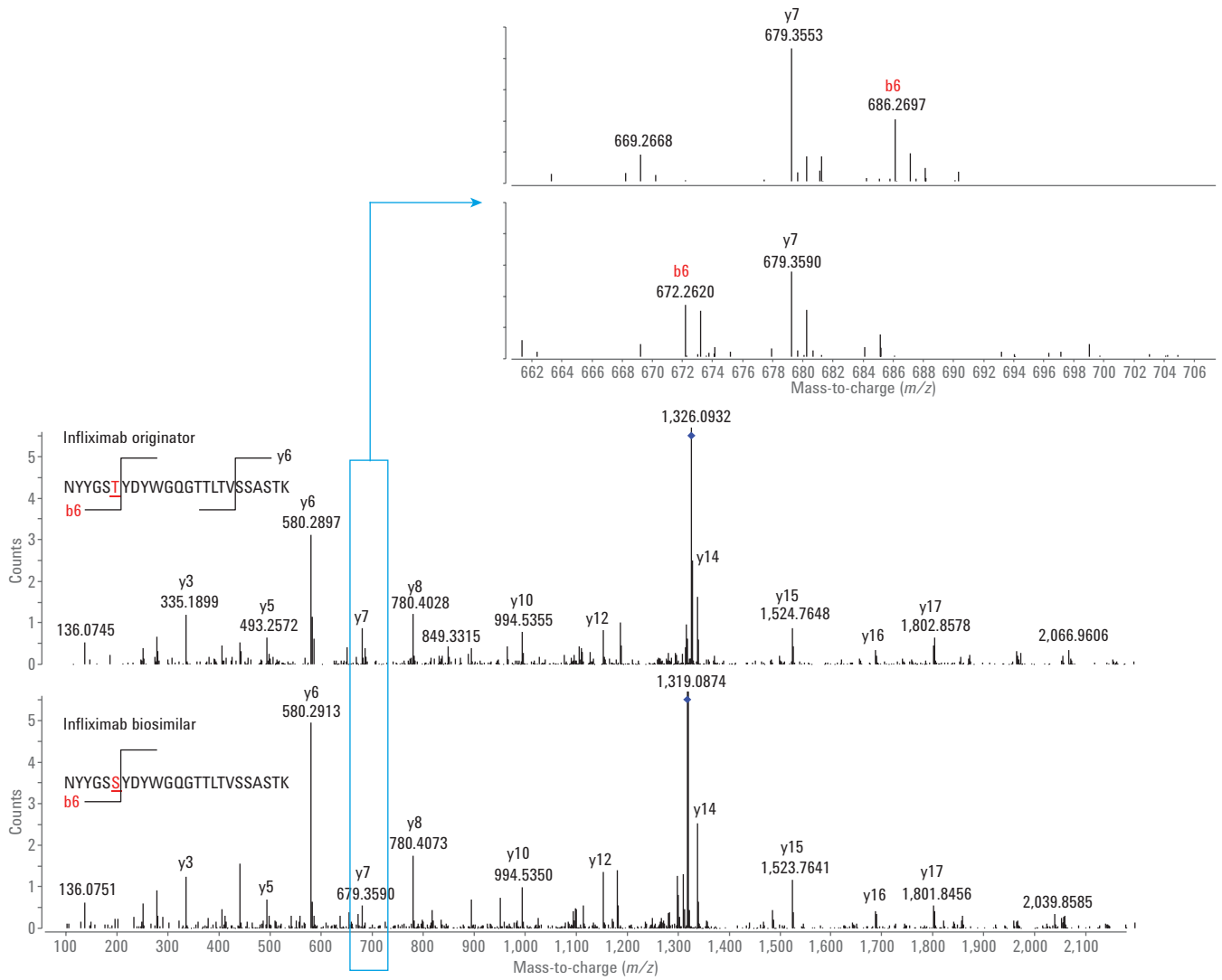


Figure 3. LCxLC/MS/MS data acquired on-the-fly in data-dependent mode, confirming the threonine to serine substitution.



Figure 4 displays the LC×LC tryptic peptide maps of infliximab originator and candidate biosimilar generated on the combination SCX×RPLC. From these measurements, the same conclusions can be drawn, yet the chromatographic behavior of the differential spots is altered. The peptide SLSLSPGK separates out from the peptides SLSLSPG and SLSLSPGI on the first SCX dimension as a result of the net +1 charge (at pH 3) of the former peptide, as opposed to the net 0 charge (at pH 3) of the latter two peptides. Again, a minor retention difference is noticed for peptides NYYGSTYDYWGQGTTLVSSASTK and NYYGSSYDYWGQGTTLVSSASTK. Note that a shorter method was used in this case.

Observed mutations were confirmed with reverse transcription polymerase chain reaction (RT-PCR) and DNA sequencing, as illustrated in Figure 5, for a CHO clone producing a candidate biosimilar with the correct sequence, and with the incorrect sequence.

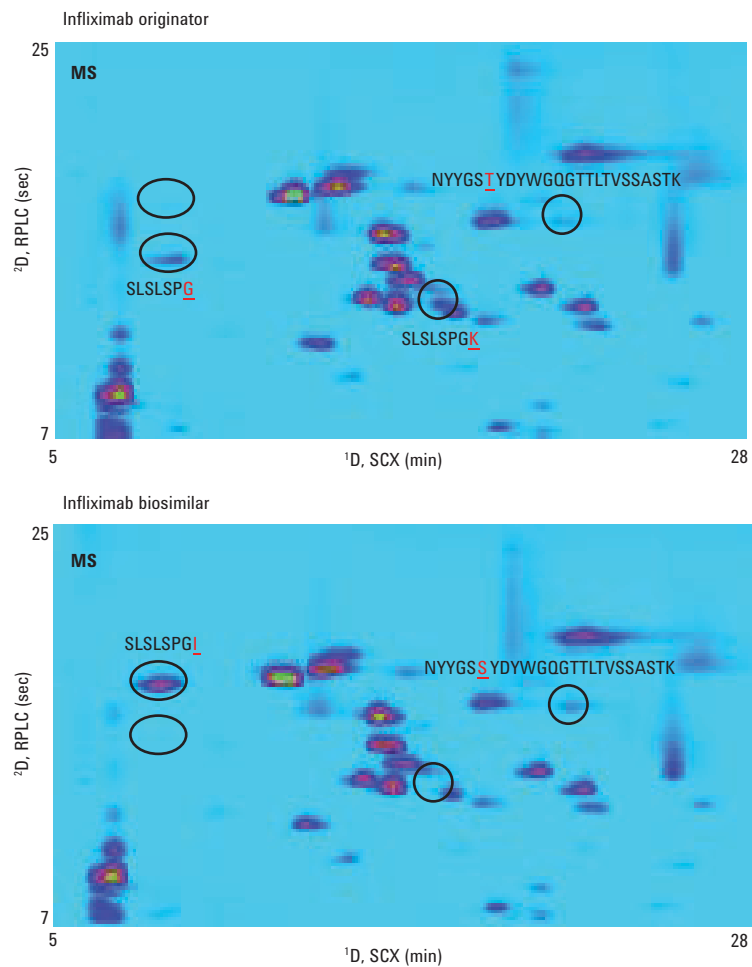


Figure 4. SCX×RPLC/Q-TOF MS analysis of infliximab originator and candidate biosimilar.

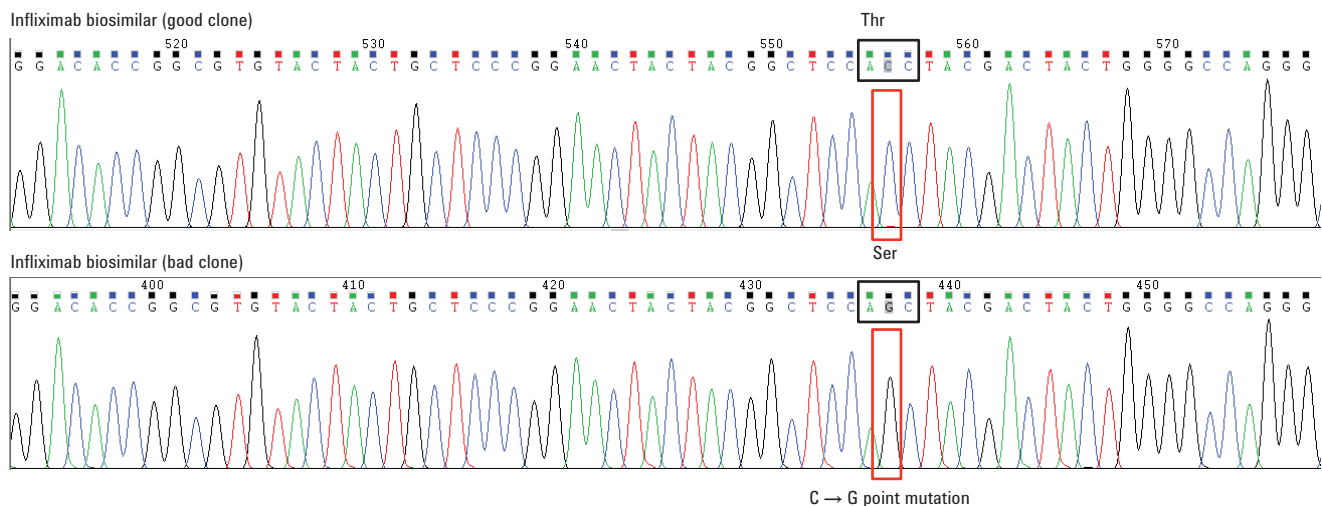


Figure 5. RT-PCR and DNA sequencing of CHO clones expressing the correct (top) and incorrect (bottom) infliximab sequence, illustrating the C-to-G point mutation and, thus, the threonine-serine substitution in the latter case.

## Conclusion

The Agilent 1290 Infinity II 2D-LC solution, combined with an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight LC/MS, successfully enabled pinpointing critical differences between an infliximab originator and candidate biosimilar.

A difference in the amino acid sequence of the heavy chain is revealed that *a priori* excludes this candidate biosimilar for further development since authorities require the amino acid sequence to be identical.

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