2D-LC/MS Characterization of Charge Variants Using Ion Exchange and Reversed-Phase Chromatography

Multiple Heart-Cutting 2D-LC Analysis of Innovator versus Biosimilar Monoclonal Antibodies

Suitable for Agilent
1290 Infinity III LC

Application Note

Biotherapeutics and Biologics

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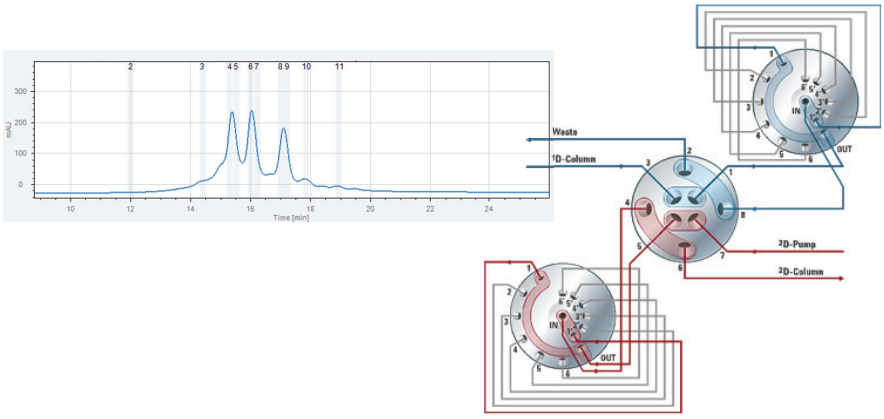
Abstract

The determination of charge variants is one of the most important quality attributes for the characterization of biopharmaceuticals such as monoclonal antibodies (mAbs). Peak identification using mass spectrometric analysis can be challenging mostly due to the incompatibility of the mass spectrometer with the buffers used during ion exchange chromatography. With the Agilent 1290 Infinity 2D-LC solution, the second dimension enables automated desalting as well as denaturation by the addition of reversed-phase (RP) chromatography for a straightforward online LC/MS workflow. This Application Note demonstrates the analysis of charge variants of Rituximab (both innovator and biosimilar molecules) using multiple heart-cutting 2D-LC with MS detection.



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Introduction

The market demand for biopharmaceuticals, especially monoclonal antibodies (mAbs) has been rapidly increasing. mAbs represent a class of highly advanced, but expensive, pharmaceutical products. These circumstances led to the development of less costly biosimilars of innovator biopharmaceuticals[1]. During the production process, mAbs are subjected to several posttranslational modifications (PTMs), for example, oxidation, deamidation, glycosylation, or lysine truncation. These PTMs affect the biochemical and biophysical properties of the mAb regarding activity, efficacy, or stability. Hence, comparability studies focusing on quality attributes are of the utmost importance to guarantee product equivalence. One important quality attribute is the charge variant profile, which can be determined using ion exchange chromatography (IEX). Generally, the separation of charge variants can be achieved using either salt or pH gradients. To qualify the charge variant pattern of the mAbs, further analysis is necessary, for example, by identification with mass spectrometry (MS). However, identification using online MS analysis after IEX is not a straightforward procedure, mostly due to the incompatibility of the most common ion exchange buffers (phosphate buffers containing sodium chloride) with the electrospray ionization process.

The Agilent 1290 Infinity 2D-LC solution enables automated desalting, and offers denaturation and, if required, additional separation by the addition of reversed-phase chromatography (RP) in the second dimension. Using multiple heart-cutting 2D-LC, heart-cuts from the first dimension are stored in the 40 µL loops of two 6-position/14-port valves, for a subsequent desalting/denaturation step in the second dimension before introduction into the MS.

This Application Note demonstrates charge variant analysis of Rituximab innovator and biosimilar using multiple heart-cutting 2D-LC analysis with MS detection. Three ion exchange gradients (two salt gradients and one pH gradient) were compared for the first dimension separation and evaluated regarding RT precision, resolution, and MS compatibility.

Experimental

Instrumentation

The Agilent 1290 Infinity 2D-LC solution was composed of the following modules:

- Agilent 1290 Infinity Binary Pumps (2 × G4220A)
- Agilent 1290 Infinity Autosampler (G4226A) with 1290 Infinity Thermostat (G1330B)
- Agilent 1290 Infinity Thermostatted Column Compartments (2 × G1316C)
- Agilent 1290 Infinity valve drive (G1170A) with 2-position/4-port duo-valve (2D-LC valve head, G4236A)
- Agilent 1290 Infinity valve drives (2 × G1170A) with multiple heart-cutting valves (2 × G4242-64000) equipped with 40-µL loops
- Agilent 1290 Infinity Diode Array Detectors (2 × G4212A) with 10 mm Max-Light cartridge cell (G4212-60008)
- Agilent 6530 Accurate Mass QTOF LC/MS system

When phosphate buffers were used for IEX, an Agilent 1260 Infinity Bio-Inert Quaternary Pump (G5611A) was used in the first dimension.

Columns

- Agilent Bio mAb, nonporous, 2.1 × 250 mm, 5 µm, PEEK (p/n 5190-2411)
- AdvanceBio RP-mAb C4, 2.1 × 75 mm, 3.5 µm (p/n 797775-904)

Software

- Agilent OpenLab CDS ChemStation Edition Software, version C.01.07 [27] with Agilent 1290 Infinity 2D-LC Acquisition Software, version A.01.02 [24]
- Agilent MassHunter Workstation Software, Version B.05.01, Build 4.0.479.0
- Agilent Buffer Advisor A.01.01 [009]

Solvents

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). Acetonitrile was purchased from Merck (Darmstadt, Germany). Ammonium formate, sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride, and formic acid (FA) were purchased from Sigma-Aldrich, St. Louis, USA.

Chromatographic conditions

Multiple heart-cutting 2D-LC analysis IEX/RP	
Valve and loop configuration	2-position/4-port duo 2x6 loops (cocurrent, Figure 4) Loop size 40 µL
Gradient 1	Salt gradient 0 to 200 mM NaCl in phosphate buffer pH 6.2 (quaternary gradient) using an Agilent 1260 Infinity Bio-inert Quaternary Pump in first dimension (calculated with Agilent Buffer Advisor)
1D mobile phase	A: Water B: 1,700 mM NaCl C: 30 mM NaH_2PO_4 D: 60 mM NaH_2PO_4
1D flow rate	0.25 mL/min
1D gradient	0 minutes: 7.59 %A, 0.0 %B, 84.82 %C, 7.59 %D 30 minutes: 1.31 %A, 11.8 %B, 73.85 %C, 13.08 %D 35 minutes: 1.31 %A, 11.8 %B, 73.85 %C, 13.08 %D
Gradient 2	pH gradient with phosphate buffer pH 6.8 to 8.2 using an Agilent 1260 Infinity Bio-inert Quaternary Pump in the first dimension (calculated with Agilent Buffer Advisor)
1D mobile phase	A: Water B: 1,700 mM NaCl C: 30 mM NaH_2PO_4 D: 60 mM NaH_2PO_4
1D flow rate	0.25 mL/min
1D gradient	0 minutes: 21.54 %A, 0.0 %B, 56.91 %C, 21.55 %D 14.09 minutes: 39.19 %A, 0.0 %B, 21.62 %C, 39.19 %D 21.21 minutes: 44.37 %A, 0.0 %B, 11.26 %C, 44.37 %D 30 minutes: 47.67 %A, 0.0 %B, 4.66 %C, 47.67 %D 35 minutes: 47.67 %A, 0.0 %B, 4.66 %C, 47.67 %D
Gradient 3	Salt gradient with ammonium formate at pH 6.2 as MS-compatible buffer conditions using an Agilent 1290 Infinity Binary Pump in both dimensions
1D mobile phase	A: 10 mM ammonium formate pH 6.2 B: 500 mM ammonium formate, pH 6.2
1D flow rate	0.2 mL/min
1D gradient	0 minutes: 0 %B 30 minutes: 40 %B 35 minutes: 100 %B 38 minutes: 0 %B

Gradients 1, 2, and 3	
1D stop time	75 minutes
2D mobile phase	A: Water + 5 %FA B: Acetonitrile with 5 %FA
2D parameter mode	Heart-cutting
2D gradient stop time	3.0 minutes
2D cycle time	4.5 minutes
Flow	1 mL/min
Idle flow	0.10 mL/min
2D gradient	0.0 minutes: 10 %B 2.5 minutes: 60 %B 2.75 minutes: 90 %B
2D time segments	Heart cutting time points for 3 Ammonium formate salt gradient as example timetable 11.90 minutes: sampling time 0.20 minutes 14.30 minutes: sampling time 0.20 minutes 15.20 minutes: sampling time 0.20 minutes 15.42 minutes: sampling time 0.20 minutes 15.90 minutes: sampling time 0.20 minutes 16.12 minutes: sampling time 0.20 minutes 16.90 minutes: sampling time 0.20 minutes 17.12 minutes: sampling time 0.20 minutes 17.75 minutes: sampling time 0.20 minutes 18.85 minutes: sampling time 0.20 minutes
Injection volume	6 µL
Thermostat autosampler	10 °C
1D column temperature	22 °C
2D column temperature	70 °C
Diode array detectors	280 nm, 4 nm, Ref. 360 nm, 100 nm Peak width 1D: 0.025 minutes (0.5 seconds response time) (10 Hz) Peak width 2D: > 0.0063 minutes (0.13 seconds response time) (40 Hz)

MS parameters

Parameter	Value
Reference mass	1,821.9523 Da
Gas temperature	300 °C
Sheath gas temperature	400 °C
Gas flow	13 L/min
Sheath gas flow	12 L/min
Nebulizer	45 psi
V_{cap}	5,000 V
Nozzle	2,000 V
Fragmentor	170
Skimmer	65 V
Oct 1 RF V_{pp}	750
Mode	MS

Diverter valve timetable after IEX with phosphate/salt buffer (1)

Time segment no.	Start time (min)	Diverter valve position
1	0	Waste
2	19.2	MS
3	20.2	Waste
4	25.6	MS
5	26.6	Waste
6	30.1	MS
7	31.1	Waste
8	34.6	MS
9	35.6	Waste
10	39.1	MS
11	40.1	Waste
12	43.6	MS
13	44.6	Waste
14	49.9	MS
15	50.9	Waste
16	54.4	MS
17	55.4	Waste
18	58.9	MS
19	59.9	Waste
20	63.4	MS
21	64.4	Waste
22	67.9	MS
23	68.9	Waste

Results and Discussion

Rituximab biosimilar and innovator charge variants were analyzed with weak cation exchange chromatography (WCX) using a flat salt gradient in ammonium formate buffer (gradient 3), (Figure 1). Only one main peak was visible for the innovator, while three prominent peaks were found for the biosimilar. The main peak of the innovator eluted at the same time as the first of the three main peaks of the biosimilar. To qualify the identity of the three biosimilar peaks in comparison to the single main peak of the innovator, further analysis is necessary, for example, by identification with MS. Regarding subsequent MS analysis, the first dimension was optimized for MS compatibility as well as resolution and retention time reproducibility.

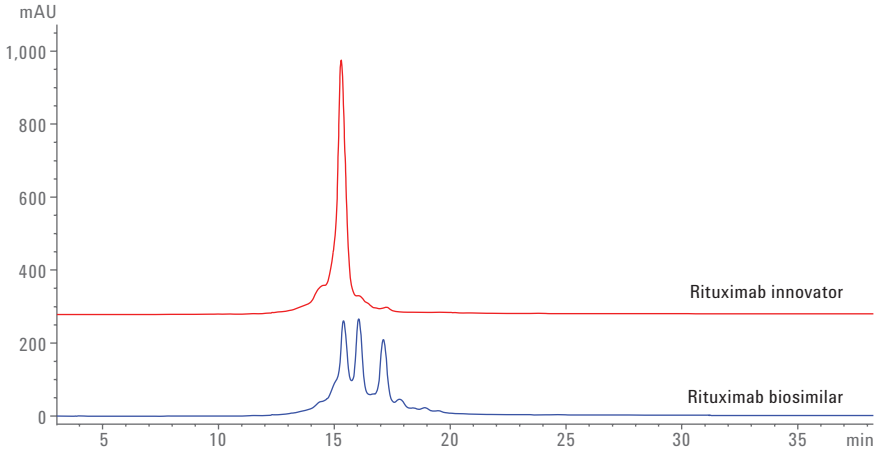

Figure 1. Charge variant analysis of rituximab innovator (red) and biosimilar (blue) using salt gradient with ammonium formate (gradient 3). There is a clear difference visible in the charge pattern for the rituximab biosimilar compared to the innovator.

First-dimension method optimization

Two salt gradients and one pH gradient were evaluated using the Rituximab biosimilar regarding resolution, reproducibility, and MS compatibility to find the most suitable method for the first dimension. Traditionally, salt gradients (mostly NaCl) in phosphate buffer systems are used for the separation of mAb charge variants in the range of pH 6 to 8. However, in previous Application Notes, it was demonstrated that the separation power of pH gradients can be higher compared to standard salt gradients using, for example, NaCl[2,3].

All three buffer systems revealed advantages and disadvantages. The most stable buffer system with highly reproducible RTs was the salt gradient in phosphate buffer (Figure 2A), followed by the phosphate pH gradient (Figure 2B) and the ammonium formate salt gradient (Figure 2C). However, both the phosphate pH gradient as well as the ammonium formate salt gradient showed a higher resolution of the three main peaks as well as additional peaks. The biggest advantage of the ammonium formate salt gradient though, is the volatile nature of this mobile phase[4]. It is compatible with online MS analysis without immense ion suppression or contaminated MS source components. In contrast, if phosphate buffer is introduced into the MS, it can lead to massive phosphate adduction as well as ion suppression. In addition, the introduction of phosphate buffer/NaCl into the MS leads to substantial salt layer formations on ESI source components. However, in many already established ion exchange methods, phosphate with NaCl is the preferred type of buffer. To keep the already established methods for the first-dimension separation, a timetable can be created using the diverter valve of the MS to prevent any buffer residues from being introduced into the MS source. As the salt is eluted with the column dead time from the second-dimension column, it is possible to set up an MS time table to switch the diverter valve so that no salt reaches the MS source, and is directly led into waste.

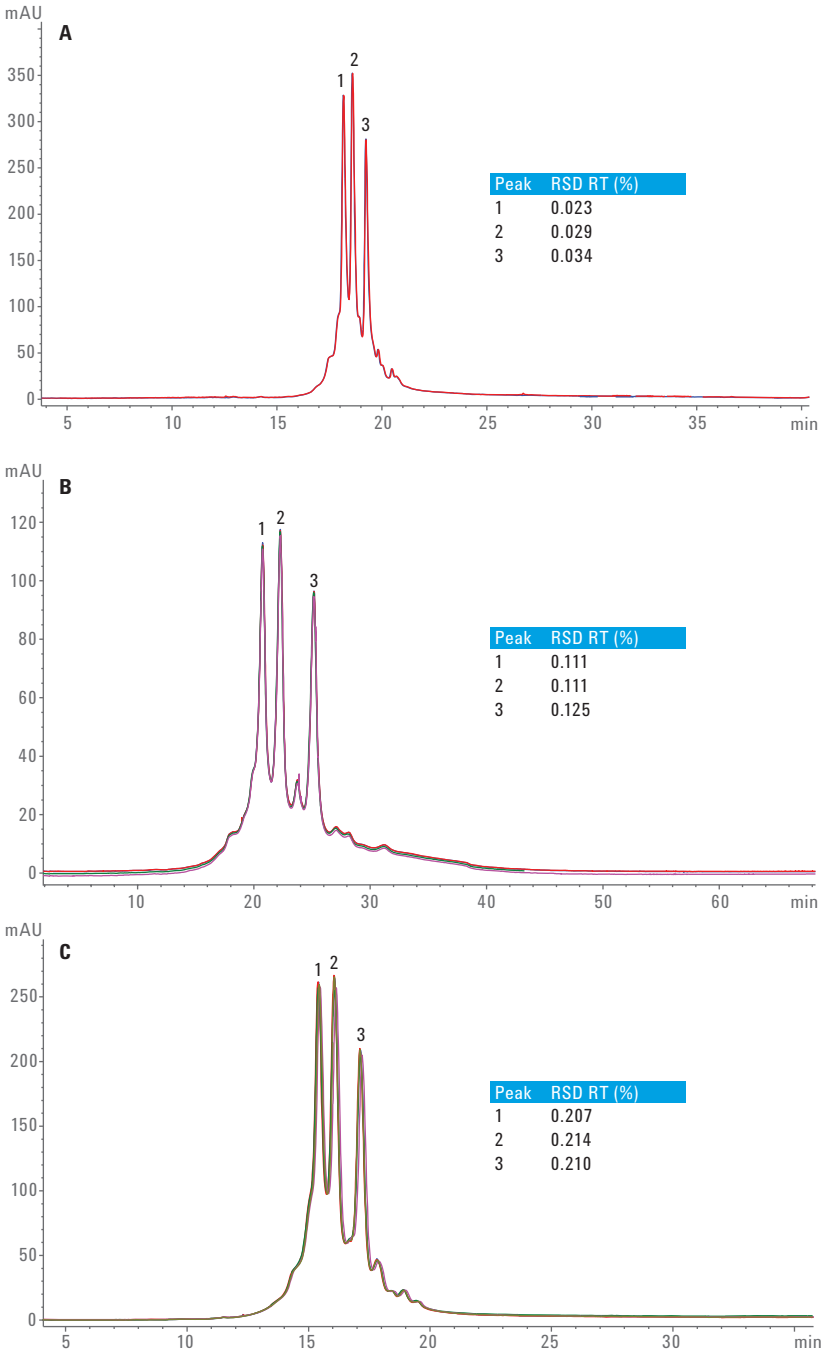

Figure 2. Analysis of rituximab biosimilar with WCX using three different buffer systems: A) Phosphate buffer at pH 6.2 with flat salt gradient, B) phosphate buffer with pH gradient between 6.8 and 8.2, and C) Ammonium formate buffer at pH 6.2 with flat salt gradient. For all buffer systems, the RT time precision is given.

2D-LC with WCX in the first dimension and RP in the second dimension

In this Application Note, the second dimension is not primarily used for a further orthogonal separation dimension, but is rather regarded as a desalting step for intact mAbs to remove buffer residues from the first dimension using RP chromatography. The transfer of the mAb into a denaturing RP system (acidified water and acetonitrile) enables straightforward intact protein MS analysis. This is also true if ammonium formate (although a volatile, MS-compatible buffer) is used in the first dimension, as native MS analysis entails complex and time-consuming MS method development[5]. The combination of ion exchange chromatography as a completely water-based solvent system with RP in the second dimension is perfectly compatible. Regarding the peak pattern of the charge variants in the first dimension, multiple heart cutting 2D-LC is the method of choice to transfer selected peak areas to the RP column in the second dimension. In this case, the three main peaks of the biosimilar were chosen for MS analysis after RP in the second dimension.

Using the preview option in the 2D pump setup, a previously recorded first-dimension chromatogram was loaded, and the peaks intended for the second-dimension analysis were chosen.

Figure 3 shows the 2D pump setup with the first-dimension chromatogram at the bottom. The pale orange bars on the chromatogram mark the 2D time segments where the peaks are sampled by heart-cutting, also visible in the table above the chromatogram (red box). Due to the multiple heart-cutting setup using 12 40-µL loops in two 6-position/14-port valves (Figure 4), it is possible to park peaks from the first dimension, enabling longer second-dimension gradients compared to those possible using comprehensive 2D-LC. For more detailed information about the setup, the mode of operation, and software features of multiple heart-cutting 2D-LC analysis see references[6,7].

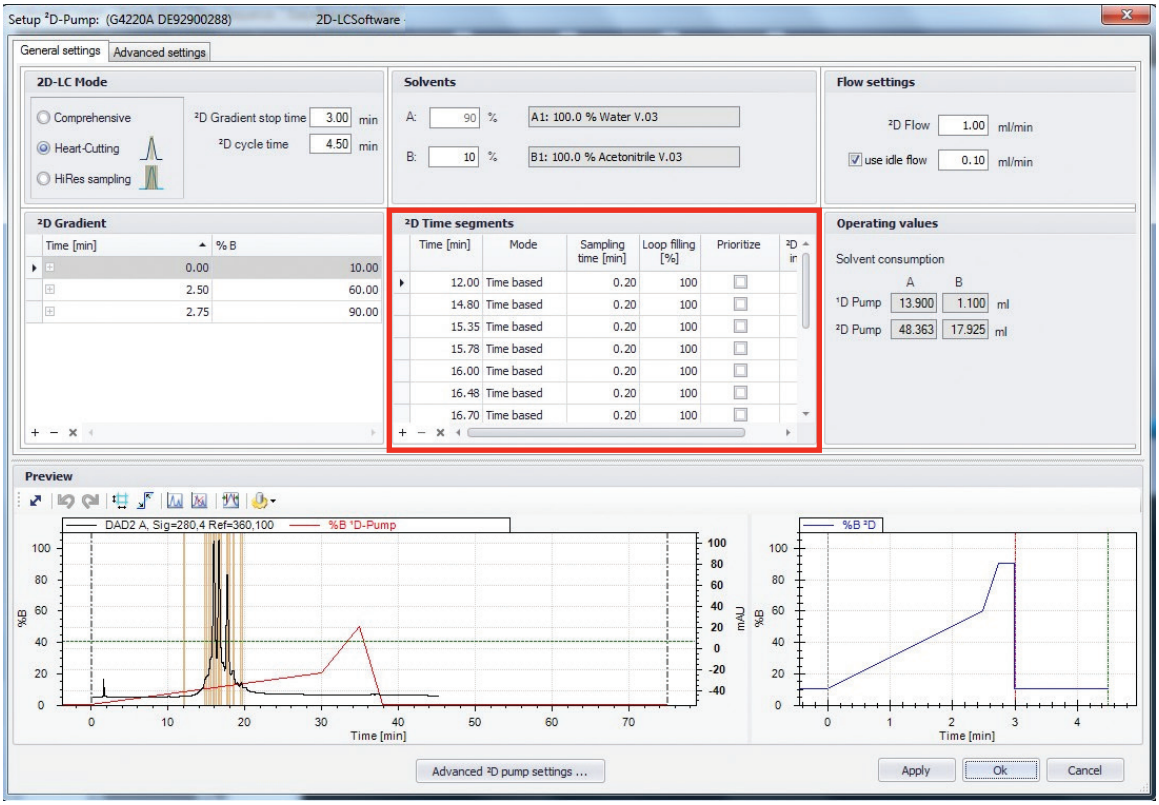

Figure 3. 2D pump setup for multiple heart-cutting method.

Figure 5 shows the results of the multiple heart-cutting 2D-LC analysis (using ammonium formate in the first dimension) presented in the 2D-LC heart-cut viewer. In the top two panels, the first-dimension chromatogram (left) with the marked heart-cuts and the heart-cut table (right) is shown. At the bottom, the second-dimension chromatogram as recorded by the second-dimension DAD is shown on the left. Here, the order of second-dimension analyses of the heart-cuts with analysis in reversed storage order and flush gradients (F) can be retraced. On the right, single second-dimension chromatograms can be displayed. As the second dimension in this case is mainly regarded as cleanup step, only one main peak is observed.

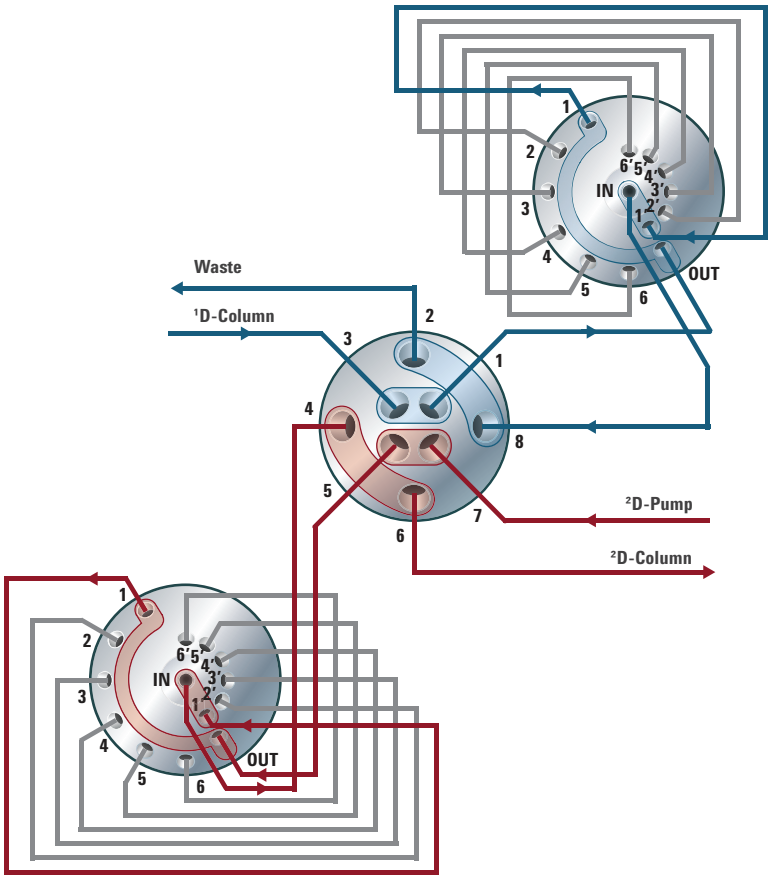

Figure 4. Plumbing diagram of the 2-position/4-port-duo valve with two 6-position/14-port valves with 12 preinstalled 40 µL loops.

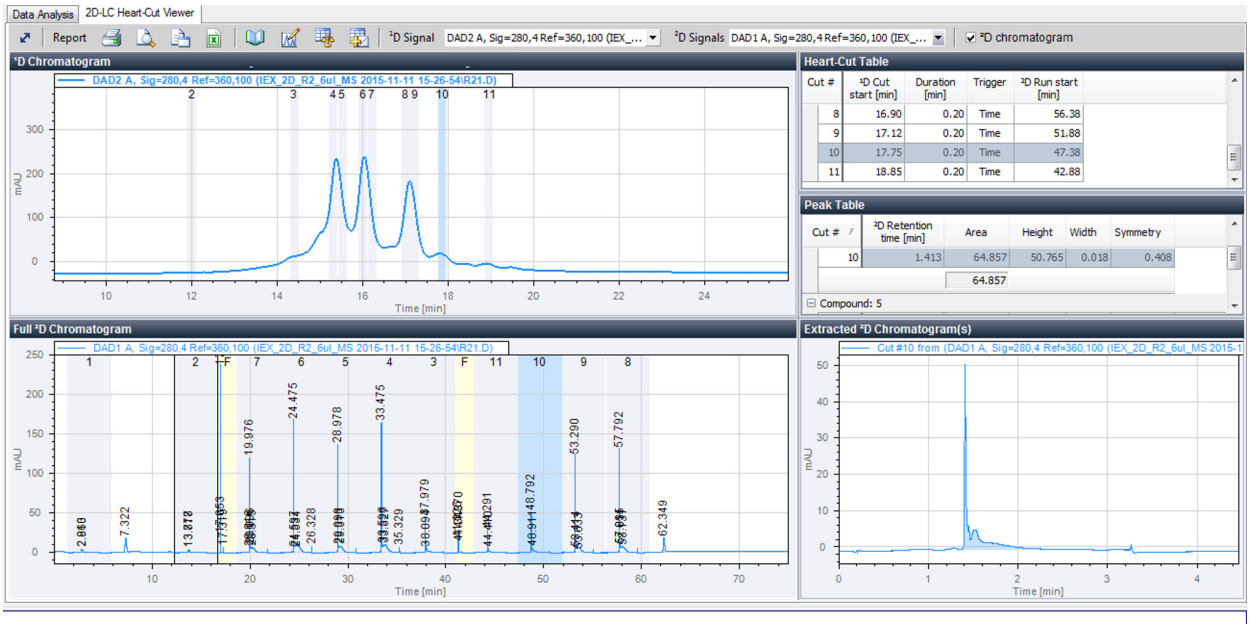

Figure 5. Results of the multiple heart-cutting 2D-LC analysis presented in the 2D-LC heart-cut viewer.

Online MS detection

The second-dimension effluent was also introduced into the Agilent 6530 Accurate Mass Q-TOF LC/MS system to determine the intact protein mass of the main peaks. The flow was split 1:4 for the introduction from the second dimension into the MS and the second dimension DAD. As ammonium formate buffer was used in the first dimension, no diverter valve table was necessary. Figure 6 shows the Gaussian distribution of the charge state envelope (Figure 6A) as well as the deconvoluted spectrum (Figure 6B) for innovator Rituximab. The spectra were deconvoluted using the maximum entropy deconvolution algorithm in Agilent MassHunter BioConfirm Software. The deconvoluted spectrum of the intact innovator Rituximab shows multiple mass peaks corresponding to different glycoforms of the mAb, for example, G0F/G0F.

As described in a previous section, the main peak of the innovator molecule eluted at the same retention time as the first of the three main peaks of the biosimilar (Figure 1). This leads to the assumption that the identity of the first main peak of the biosimilar is the same as the main peak of the innovator. MS Q-TOF analysis enabled the qualification of the three different main peaks found in the charge variant pattern of the biosimilar. Figure 7 shows the deconvoluted spectra of charge variant main peaks 1–3 (Figures 7A–C). The mass of the first biosimilar main peak for G0F/G0F is 147,082 Da, identical to G0F/G0F of the innovator. The G0F/G0F of the second (Figure 7B) and third (Figure 7C) charge variant peak are shifted with an interval of about 128 Da, representing the mass of lysine, leading to the assumption that the three main peaks are C-terminal lysine variants. The latter was confirmed by C-terminal digestion by Carboxypeptidase B (data not shown). C-terminal lysine variants are commonly observed in monoclonal antibodies. Although no substantial influence on the bioactivity of the mAbs has been observed, they reflect the consistency of the manufacturing process[8].

The 2D multiple heart-cutting analysis was also repeated using a phosphate-buffered salt gradient as a well-established and highly reproducible buffer system in the first dimension (MS incompatible system), followed by reversed-phase cleanup in the second dimension. The analysis was performed with and without the enabled time table for the MS diverter valve. In the second dimension, the mAb peak eluted between one and two minutes of the second-dimension gradient. Therefore, the LC flow was only directed into the MS during this time period. In another experiment, the flow was completely introduced into the MS source without the enabled MS time table. Figure 8 shows the deconvoluted spectra of the first main peak of the rituximab biosimilar without the MS time table enabled (A) and with the MS time table enabled (B). Both spectra look similar. No major sodium adducts were observed in both ways. Hence, the desalting step of the second dimension can be regarded as sufficient to remove salt residues from the first-dimension buffer. However, the introduction of the phosphate buffered salt gradient led to the formation of a substantial salt layer on the source surface, and required an extensive cleaning procedure even after only a few (less than 10) second-dimension gradients. Therefore, it is highly recommended to use the MS time table when such buffers are used in the first dimension.

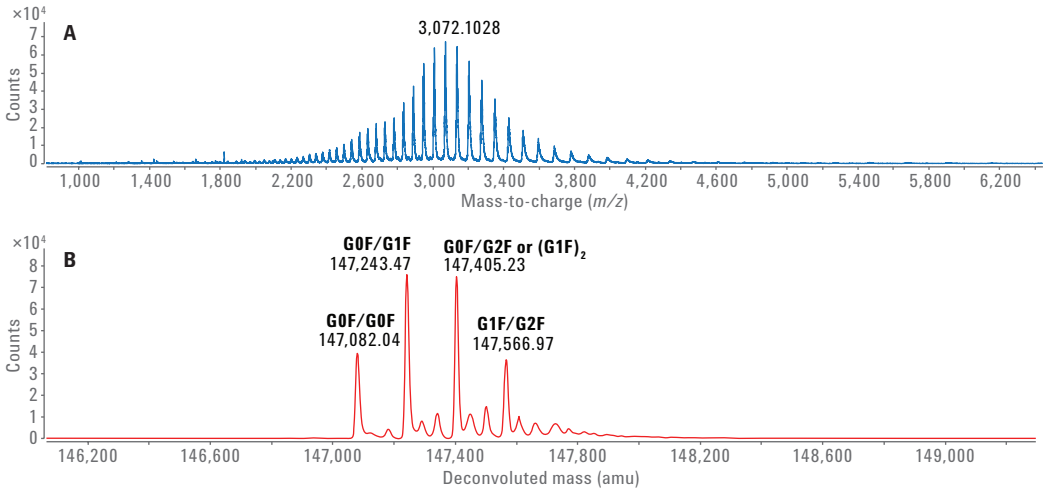

Figure 6. Intact mass analysis of innovator Rituximab using ammonium formate in the first dimension: A) Gaussian distribution of the charge envelope, B) Deconvoluted spectrum revealing different glycoforms.

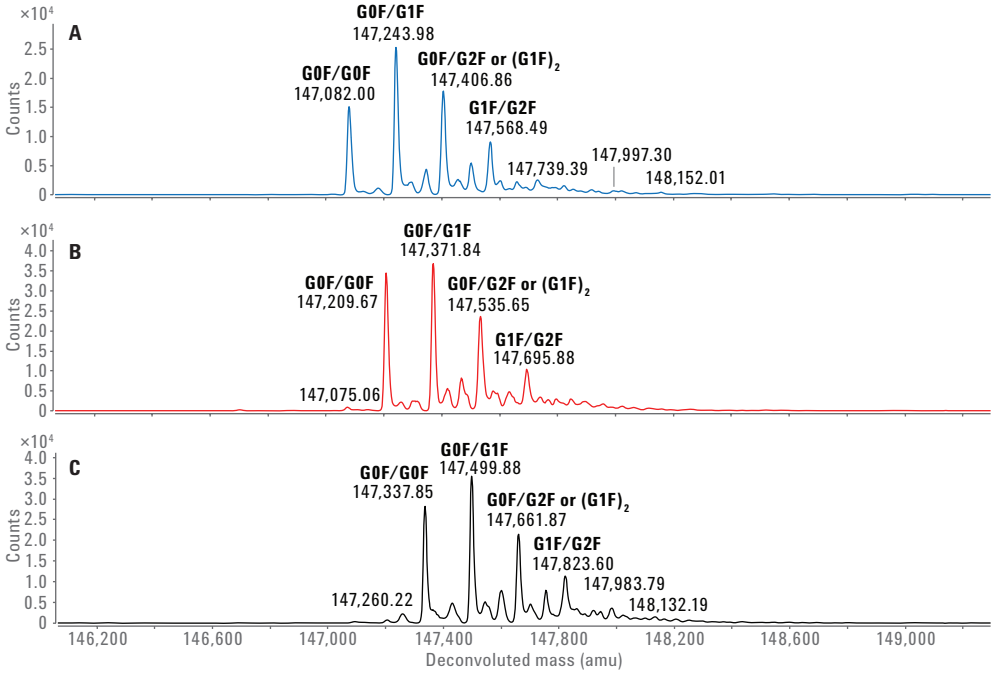

Figure 7. Deconvoluted spectra of main peak 1 (A), 2 (B), and 3 (C) of the Rituximab biosimilar using ammonium formate in the first dimension.

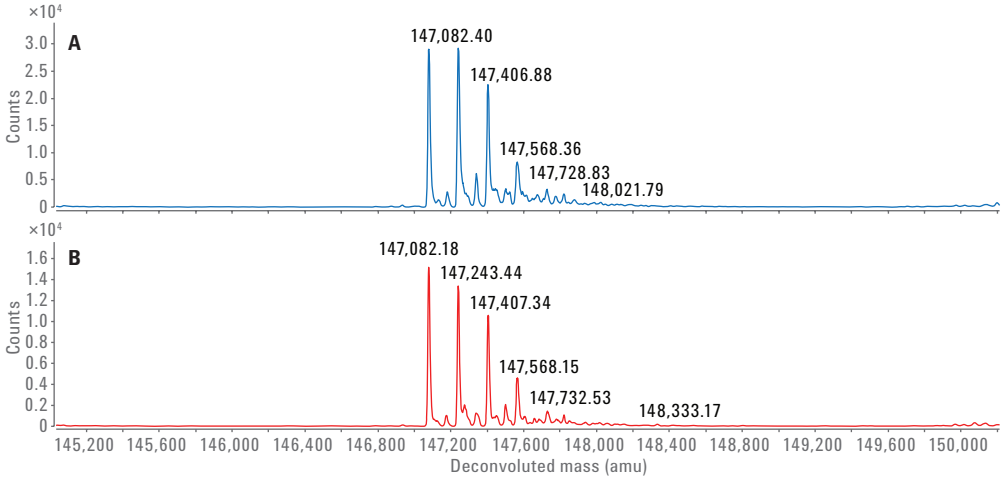

Figure 8. Deconvoluted spectra of the first main peak of rituximab biosimilar using a phosphate buffered salt gradient in the first dimension without MS time table enabled (A) and with MS time table enabled (B).

Conclusion

Innovator and biosimilar Rituximab charge variants were analyzed using weak cation exchange chromatography. To enable direct qualification of the charge pattern using online MS detection, a two-dimensional workflow was developed with RP in the second dimension. The first dimension was optimized regarding resolution and RT precision as well as MS compatibility. The best reproducibility was found for the phosphate/NaCl buffer system, whereas the highest resolution was found with the phosphate pH gradient. The ammonium formate buffer system showed good performance with a good compromise of high resolution, reproducibility, and MS compatibility. The addition of an RP second dimension enabled not only desalting (highly important after the phosphate/NaCl buffer system), but denaturation of the sample to provide straight forward MS analysis. Due to the peak pattern of three main peaks of the biosimilar in the first dimension, selected peak areas were transferred to the RP column in the second dimension using multiple heart-cutting 2D-LC. In contrast, the innovator showed only one main peak. With Q-TOF MS analysis, the mass of the first main peak of the biosimilar for G0F/G0F was found to be identical to G0F/G0F of the innovator. The G0F/G0F glycoforms of the second and third charge variants were shifted by about 128 Da, representing C-terminal lysine variants. To prevent buffer residues from entering and contaminating the MS source, an MS diverter valve time table was set up. Although no difference was seen in the MS spectra, a substantial salt layer was observed on the source after the introduction of the phosphate/NaCl buffer, requiring extensive cleaning procedures after MS analysis. Therefore, using the MS time table for switching between waste and the MS source is highly recommended when working with MS incompatible buffers.

The Agilent 1290 Infinity 2D-LC solution with multiple heart-cutting, together with the Agilent 6530 Accurate Mass Q-TOF LC/MS system represents an ideal solution for charge variant analysis of innovator and biosimilar mAbs using multiple heart-cutting 2D-LC analysis with online MS detection.

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