Online 2D-LC Characterization of Monoclonal Antibodies with Size Exclusion and Weak Cation Exchange Chromatography

Application Note

Biotherapeutics and Biologics

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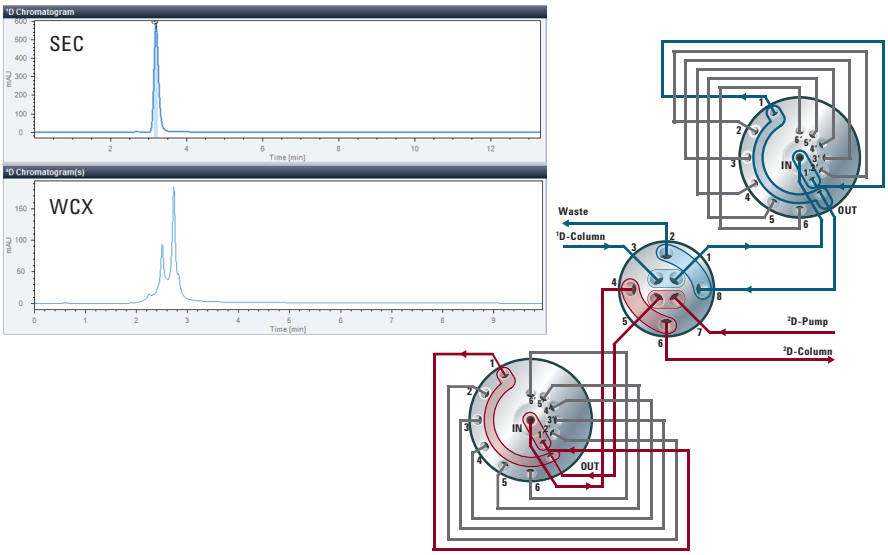
Abstract

The characterization of biopharmaceuticals can be highly complex and extensive to evaluate all important quality attributes. Online 2D-LC analysis offers a combination of different analysis methods to monitor different parameters in one run. This Application Note shows a combination of aggregate analysis with size exclusion chromatography in the first dimension, followed by ion exchange chromatography for charge variant profiling in the second dimension. Heart-cutting 2D-LC enables online transfer of peaks from the first dimension to the second dimension, allowing highly precise aggregate analysis and charge variant profiling within 13 minutes, and offering a combined online analysis of two of the most important quality attributes. In addition, high-resolution sampling 2D-LC enables the detection of coeluting compounds and delivers accurate 2D-LC quantification by the transfer of the entire 1D peak area to the second dimension.



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Introduction

Detailed and in-depth characterization of biopharmaceuticals is of extreme importance to ensure their safety and efficacy from triggering unpredictable immunogenic responses. Different parameters have to be monitored during various stages in the development and production of the product. In early phases of process development, an enormous number of samples need to be screened. Those extensive studies include important quality attributes such as titer analysis, aggregation studies, charge variant and glycan profiling, peptide mapping, and many others. Due to the extent and complexity of the used methods, full characterization of biopharmaceuticals is an elaborative, time-consuming, and cost-intense business. Two of the most important quality attributes in monoclonal antibodies (mAbs) are aggregation and charge variant analysis[1].

With the Agilent 1290 Infinity II 2D-LC solution, two analysis types can be combined in an online setup to increase efficiency and reduce hands-on time (no fraction collection and reinjection necessary). In addition to the two classical 2D-LC modes: (multiple) heart-cutting and comprehensive 2D-LC, acting complementary to each other, high-resolution sampling 2D-LC combines advantages of both heart-cutting and comprehensive 2D-LC. Up to 10 consecutive cuts can be defined for the first dimension with the same valve setup that is used for multiple heart-cutting 2D-LC[2]. Especially in biochromatography such as affinity chromatography, size exclusion (SEC), or ion exchange chromatography (IEX), high-resolution sampling can be helpful by enabling the analysis of larger areas of interest or broad unresolved peaks in the first dimension.

This Application Note shows the combination of aggregation analysis using SEC with weak cation exchange chromatography (WCX) for a subsequent charge variant profiling of an mAb using heart-cutting 2D-LC for a short online analysis in 13 minutes. In addition, high-resolution sampling offers the option to transfer the complete 1D peak to the second dimension to identify potential coeluting impurities as well as to enable accurate 2D-LC quantification.

Experimental

Instrumentation

The Agilent 1290 Infinity II 2D-LC Solution was comprised of the following modules:

- **First Dimension Pump**: Agilent 1260 Infinity Bio-Inert Quaternary Pump (G5611A)
- **Second Dimension Pump**: Agilent 1290 Infinity II High-Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with cooler
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2-position/4-port duo-valve (2D-LC) valve head (G4236A)
- 2× Agilent 1290 Infinity Valve Drives (G1170A) with 2x multiple heart-cutting valves (G4242-64000) equipped with 40-µL loops
- 2× Agilent 1290 Infinity II Diode Array Detectora (G7117B) with 10-mm Max-Light cartridge cell (G4212-60008)

Columns

- Agilent AdvanceBio SEC 300Å, 4.6 × 150 mm, 2.7 µm (p/n PL1580-3301)
- Agilent Bio MAb, non-porous, 4.6 × 50 mm, 1.7 µm, stainless steel (p/n 5190-2401)

Software

Agilent OpenLab CDS ChemStation Edition software, version C.01.07 [27] with Agilent 1290 Infinity 2D-LC software, version A.01.03

Solvents and samples

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). Sodium chloride and monobasic and dibasic sodium phosphate were purchased from Sigma-Aldrich, St. Louis, MO, USA.

An mAb solution was injected in a 50 mM phosphate buffer, pH 6.2 (10 mg/mL). It was filtered using an Agilent Captiva Premium Syringe Filter, regenerated cellulose membrane, 15-mm diameter, 0.45 µm pore size (p/n 5190-5109).

Results and Discussion

An mAb was analyzed for aggregates using the Agilent AdvanceBio SEC 300Å in the first dimension. Almost baseline separated, a small aggregate peak was detected in front of the main peak (Figure 1 inset). After integration, an aggregate amount of 1 % was calculated.

Chromatographic conditions

Parameter	Value
First-dimension parameters	
1D column	Agilent AdvanceBio SEC 300 Å
1D mobile phase	50 mM Phosphate buffer, pH 6.2
1D flow rate	0.5 mL/min
Second-dimension parameters	
Mode	Heart cutting and High-resolution sampling
2D column	Agilent Bio mAb
2D mobile phase	A) 25 mM Phosphate buffer, pH 6.2 B) 500 mM Sodium chloride in 25 mM phosphate buffer, pH 6.2
2D gradient stop time	6 minutes
2D cycle time	10 minutes
2D flow rate	0.5 mL/min
2D gradient	0.00 minutes – 5 % B 5 minutes – 30 % B 6 minutes – 40 % B
2D time segments	**Heart cutting** Time 1D 3.13 Mode Time based Sampling time 0.11 **High-resolution sampling** Time 1D 3.05 Mode Time based Sampling time 5 seconds Cuts 8
Injection volume	5 µL
Thermostat autosampler	6 °C
Column temperature	RT
DADs	280 nm, 4 nm, Ref. 360 nm, 100 nm
Peak widths	0.025 minutes (0.5 seconds response time) (10 Hz)

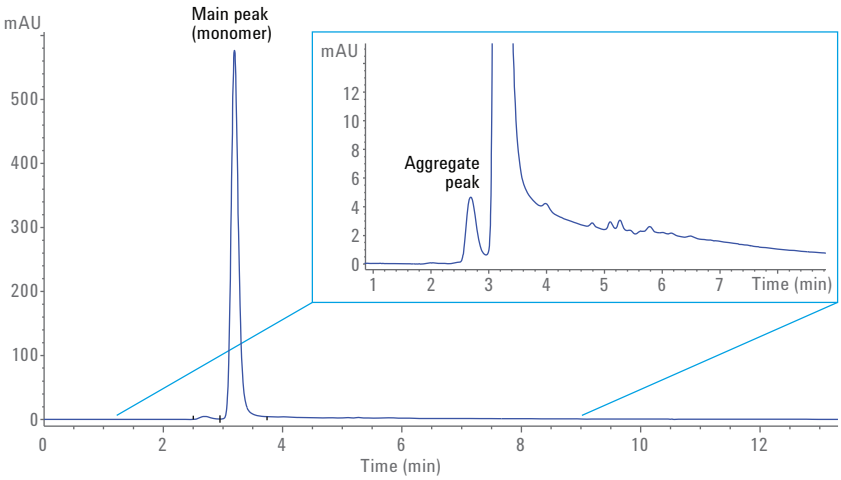

Figure 1. Aggregate analysis after size exclusion chromatography using an Agilent AdvanceBio SEC 300Å, 4.6 × 150 mm, 2.7 µm column.

A heart-cut from the main peak was transferred to the second dimension for charge variant analysis using a sub-2 µm column, the Agilent Bio MAb, nonporous, 4.6 × 50 mm, 1.7 µm for optimal resolution in a short run time. Figure 2 shows the 2D-LC Viewer with the first dimension (aggregate analysis) in the upper chromatogram, where the heart-cut that is transferred into the second dimension for charge variant analysis (lower chromatogram) is marked. In addition to the main peak, the charge variant analysis reveals two acidic and one basic variant. Acidic and basic species are defined based on their retention times (RTs) relative to the main peak. The variants eluting before the main peak in cation exchange chromatography are defined as acidic, whereas the variants eluting later than the main peak are defined as basic[3].

The short (50-mm) sub-2 µm Agilent Bio MAb column enables highly resolving charge variant analysis in the second dimension, even for shorter run times. Compared to traditional charge variant analyses with 30 to 40 minutes run times on 250-mm columns (5-µm particle size), here, a total run time of 10 minutes is enabled[4]. The cycle time for the complete 2D-LC run was approximately 13 minutes, including column regeneration.

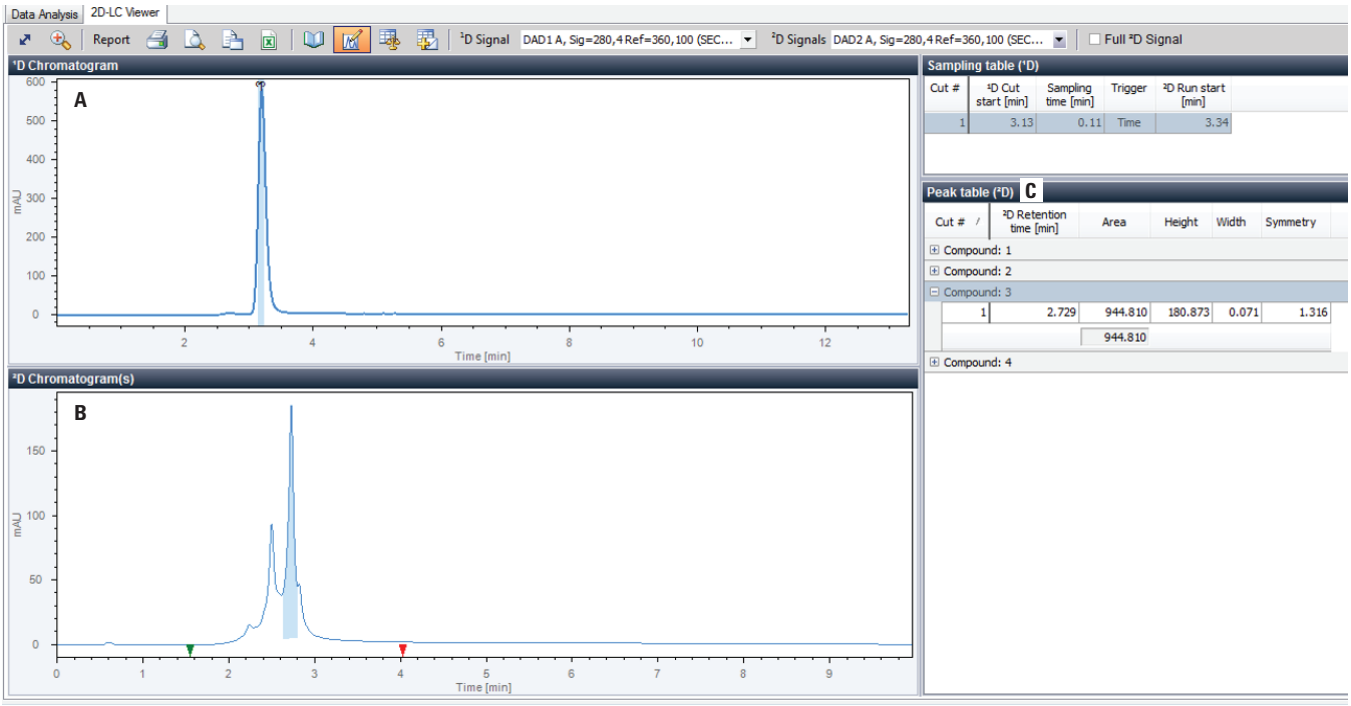

Figure 2. 2D-LC viewer with SEC aggregate analysis in the first dimension (A), and WCX charge variant analysis in the second dimension (²D signal, B). The ²D peak table (C) allows a closer look into the second dimension chromatogram. The marked compound in the ²D peak table is shown as the blue region in the ²D chromatogram. The green and the red arrows mark the integrated area in the second dimension.

Precision of retention time and area was determined in the first and second dimension for seven consecutive injections. In the first dimension, the aggregate and main peak were evaluated. Four peaks were evaluated in the second dimension: two acidic variants, one main peak, and one basic variant. Figure 3 shows an overlay of seven consecutive runs in the 2D-LC Viewer for the first and second dimension, together with the precision values for RT and peak area. For the first dimension, the RT and area precision was excellent, with < 0.08 and < 0.5 %, respectively. The second dimension precision for RT and area was also excellent, with < 0.09 and < 2.1 %, respectively.

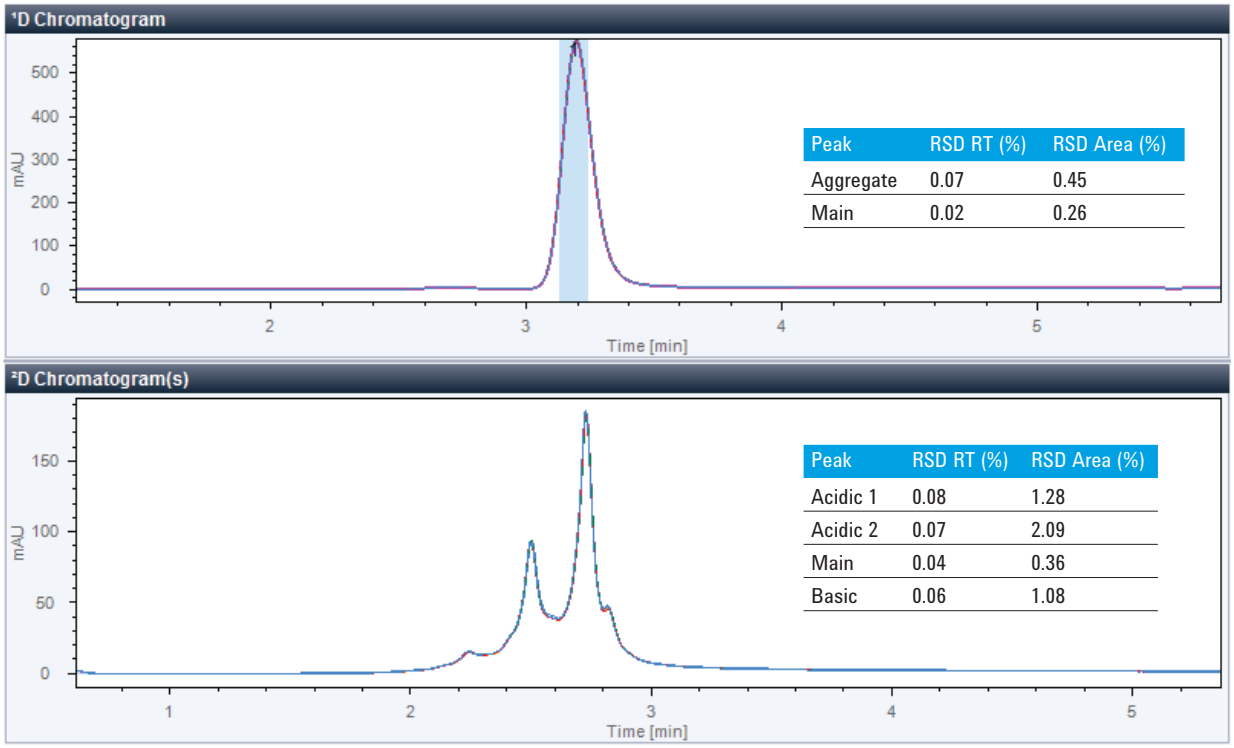

Figure 3. Overlay of seven consecutive 2D-LC runs: 1D aggregate analysis and 2D charge variant analysis.

To check for coeluting impurities after SEC analysis, the main peak was sampled into eight cuts using high-resolution sampling to include the complete peak from the first dimension (Figure 4). No coeluting impurities were detected after WCX in the second dimension, as all resulting eight chromatograms (eight cuts) were similar, only differing in signal intensity.

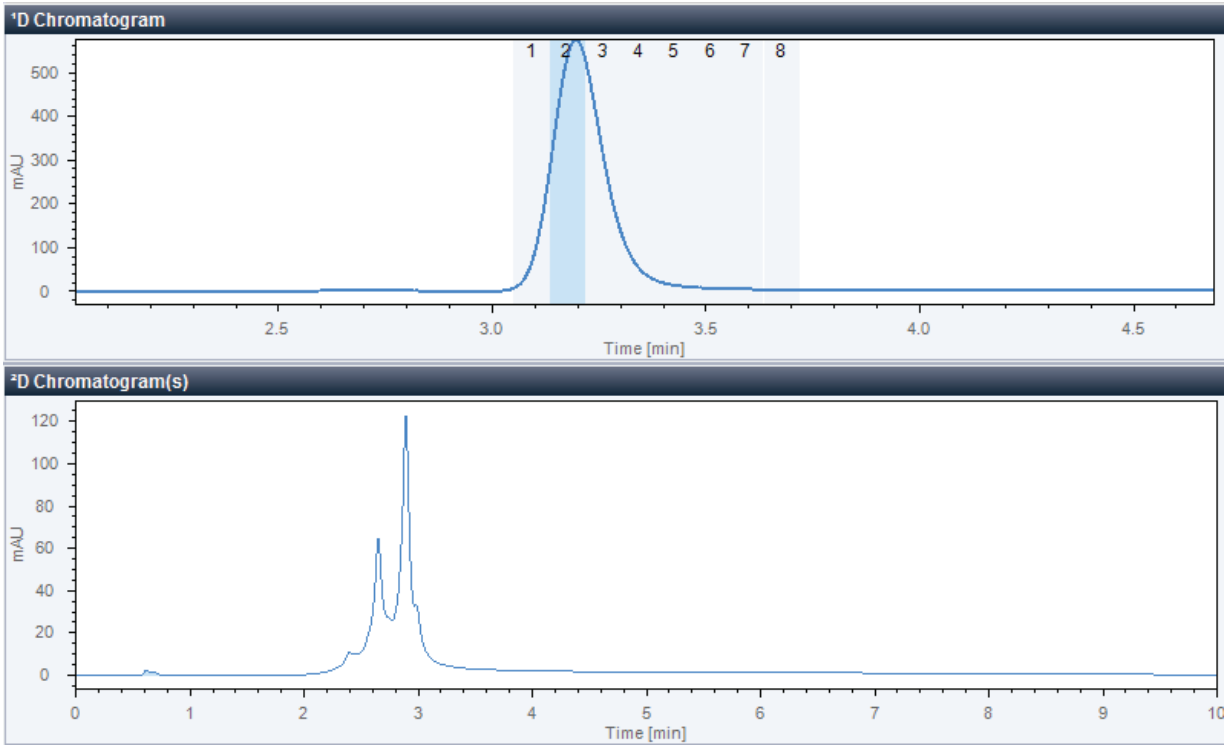

Figure 4. The 2D-LC analysis with SEC aggregate analysis in the first dimension, showing the chosen HiRes sampling set of cuts for subsequent charge variant analysis in the second dimension. No coeluting impurities were detected.

Conclusion

Aggregation and charge variant analysis were combined using the Agilent 1290 Infinity II 2D-LC solution for the analysis of mAbs. High resolution and excellent precision was found for both dimensions in a short combined SEC-WCX run within 13 minutes total cycle time using heart-cutting 2D-LC chromatography. The number of aggregates was determined to be approximately 1 % of the main peak. Charge variant analysis in the second dimension revealed two acidic and one basic variant, in addition to the main peak. Potential coeluting impurities could ideally be detected using high-resolution sampling where the relatively broad peak from the first SEC dimension was sampled in eight cuts, and transferred to the second dimension. In this case, no coeluting peaks were detected after WCX analysis in the second dimension.

References

1. ICH, ICH Topic Q6B Specifications: Test Procedures and Acceptance Criteria for Biotechnological/ Biological Products

2. Access Agilent Newsletter March 2016: http://www.agilent.com/en-us/newsletters/accessagilent/2016/mar/2D-LChrs?cid=12822

3. Du, Y.; *et al.* Chromatographic analysis of the acidic and basic species of recombinant monoclonal antibodies, *MAbs* Sep 1 **2012**, *4(5)*, 578–585.

4. Schneider, S. Simple Method Optimization in mAb Charge Variant Analysis using pH Gradients Generated from Buffer Advisor with Online pH and Conductivity Monitoring, *Agilent Technologies Application Note*, publication number 5991-3365EN, **2014**.

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