Application Note

Biopharma/Pharma

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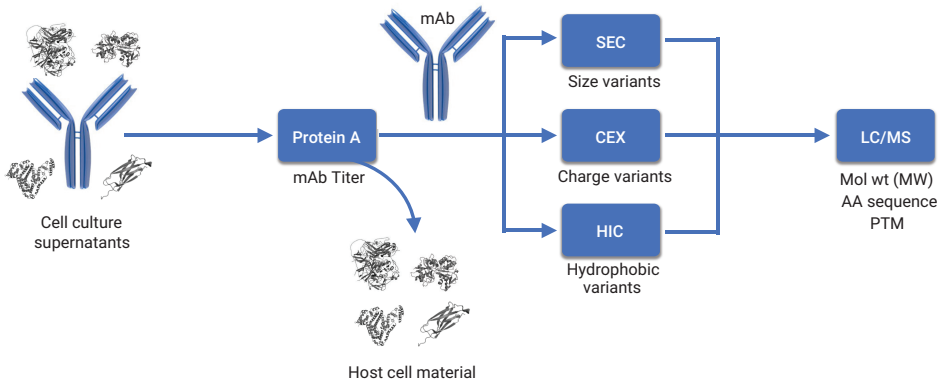
Trusted Answers

Determination of Multiple Attributes of Monoclonal Antibodies

Simultaneous and parallel multi-attribute analysis using 3D-LC/MS with ^{2}D multimethod option

Suitable for Agilent
1290 Infinity III LC



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Abstract

This application note describes multi-attribute analysis of monoclonal antibodies using the Agilent InfinityLab 2D-LC Solution and the Agilent 6530 LC/Q-TOF system. The analyzer combines Protein A affinity chromatography in the first dimension with a multimethod option (choice between SEC, CEX, or HIC) in the second dimension and desalting SEC-MS in the third dimension. This innovative 3D-LC/MS setup allows simultaneous and parallel assessment of mAb titer, size/charge/hydrophobic variants, molecular weight, amino acid (AA) sequence, and post translational modifications directly from cell culture supernatants.

Introduction

Therapeutic monoclonal antibodies (mAbs) come with a structural complexity highly demanding towards analytics. These biotechnology-derived products are composed of approximately 1,300 amino acids spread over two light and two heavy chains maintained in a functional 150 kDa tetrameric state. Amino acids can be further co- or post translationally modified, giving rise to various charge or hydrophobic variants, and the tetramer can fragment or aggregate, resulting in size variants. Ultimately, hundreds of mAb species make up the profile, safety, and efficacy of the product. Unraveling this complexity demands a wide range of analytical tools and methodologies, with liquid chromatography (LC) and mass spectrometry (MS) at the forefront.[1-3]

By combining multiple techniques in one analytical system, different structural characteristics such as molecular weight (MW), amino acid sequence, N-glycosylation, N- and C-terminal processing, deamidation, oxidation, fragmentation, and aggregation can simultaneously be assessed, a principle known as multi-attribute analysis (MAA). Two-dimensional liquid chromatography (2D-LC) operated in heart-cutting or multiple heart-cutting mode is highly promising in this respect.[4-7] This concept was recently taken to the next level by combining Protein A affinity chromatography, size-exclusion chromatography (SEC), and LC/MS in a (multiple) heart-cutting three-dimensional (^{3}D) setup.[8]

The current study further builds on this with the addition of two additional methods in the second dimension (^{2}D) – cation-exchange chromatography (CEX) and hydrophobic interaction chromatography (HIC) – and a column selector that makes it possible to define the mode used in ^{2}D (SEC, CEX, or HIC). This 3D-LC/MS multi-attribute analyzer with a multimethod option in ^{2}D allows simultaneous and parallel assessment of mAb titer, size/charge/hydrophobic variants, molecular weight, amino acid sequence, and post translational modifications directly from cell culture supernatants.

Experimental

Materials

Acetonitrile (HPLC-S), isopropanol (HPLC), water (ULC/MS), and formic acid (ULC/MS) were obtained from Biosolve (Valkenswaard, The Netherlands). NaH_2PO_4, Na_2HPO_4, NaCl, NH_4HCO_2, $(NH_4)_2SO_4$, and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Type I water was produced from tap water by an arium pro ultrapure lab water system from Sartorius (Göttingen, Germany). Humanized monoclonal antibody trastuzumab, marketed as Herceptin, was obtained from Roche (Basel, Switzerland). Trastuzumab biosimilar Chinese Hamster Ovary (CHO) cell culture supernatants were obtained from a local biotechnology company.

Sample preparation

A trastuzumab dilution series (0.02, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mg/mL) was made in 50 mM sodium phosphate pH 7.45 (Protein A mobile phase A). The trastuzumab-producing CHO clone supernatant samples were loaded on the Protein A column following centrifugation at 1,000 g for 2 minutes.

Instrumentation

An Agilent 1290 Infinity II 2D-LC System equipped with the multiple heart-cutting option, an additional Agilent 1260 Infinity II quaternary pump, two Agilent 1290 Infinity valve drives, an Agilent 2D-LC active solvent modulation (ASM) valve, an Agilent InfinityLab quick change solvent selector valve, and Agilent InfinityLab quick change 4-column selector valve were used. Connections were made with 0.12 mm id stainless steel tubing. The configuration is schematically represented in Figure 1 and detailed below. DAD detection was used in the first (Protein A) and second dimension (SEC/CEX/HIC). Additionally, an Agilent 6530 LC/Q-TOF with an Agilent Jet Stream ESI source was used for detection after the third (SEC desalting) dimension.

Configuration

^{1}D: Protein A chromatography

- Agilent 1260 Infinity II Quaternary Pump with active inlet valve (AIV) (G7111B, option 032)
- Agilent 1290 Infinity II Multisampler with Sample Thermostat (G7167B, option 101) and extension seat capillary of 80 µL (G4226-87303)
- Agilent 1290 Infinity II Multicolumn Thermostat (MCT) with valve drive installed (G7116B, option 058)
- Agilent 1290 Infinity II Diode Array Detector (G7117B) with a 3.7 mm Agilent InfinityLab Max-Light Cartridge Cell, HDR (G4212-60032)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2D-LC ASM valve head, 1,300 bar (G4243A, part number 5067-4266) with:
  - **Deck A:** 80 µL ST 0.35 mm × 831 mm M/M capillary (part number 5067-6645) installed on two transfer 0.12 mm × 170 mm M/M capillaries (part number 5500-1376) and ZDV M/M union (part number 5023-3150)
  - **Deck B:** 0.12 mm × 150 mm M/M capillary (part number 5500-1204)
  - **ASM:** 0.12 mm × 85 mm M/M capillary (part number 5500-1300)

Note: A short 3.7 mm detector flow cell was installed to reduce the signal intensity and prevent saturation of the UV signal.

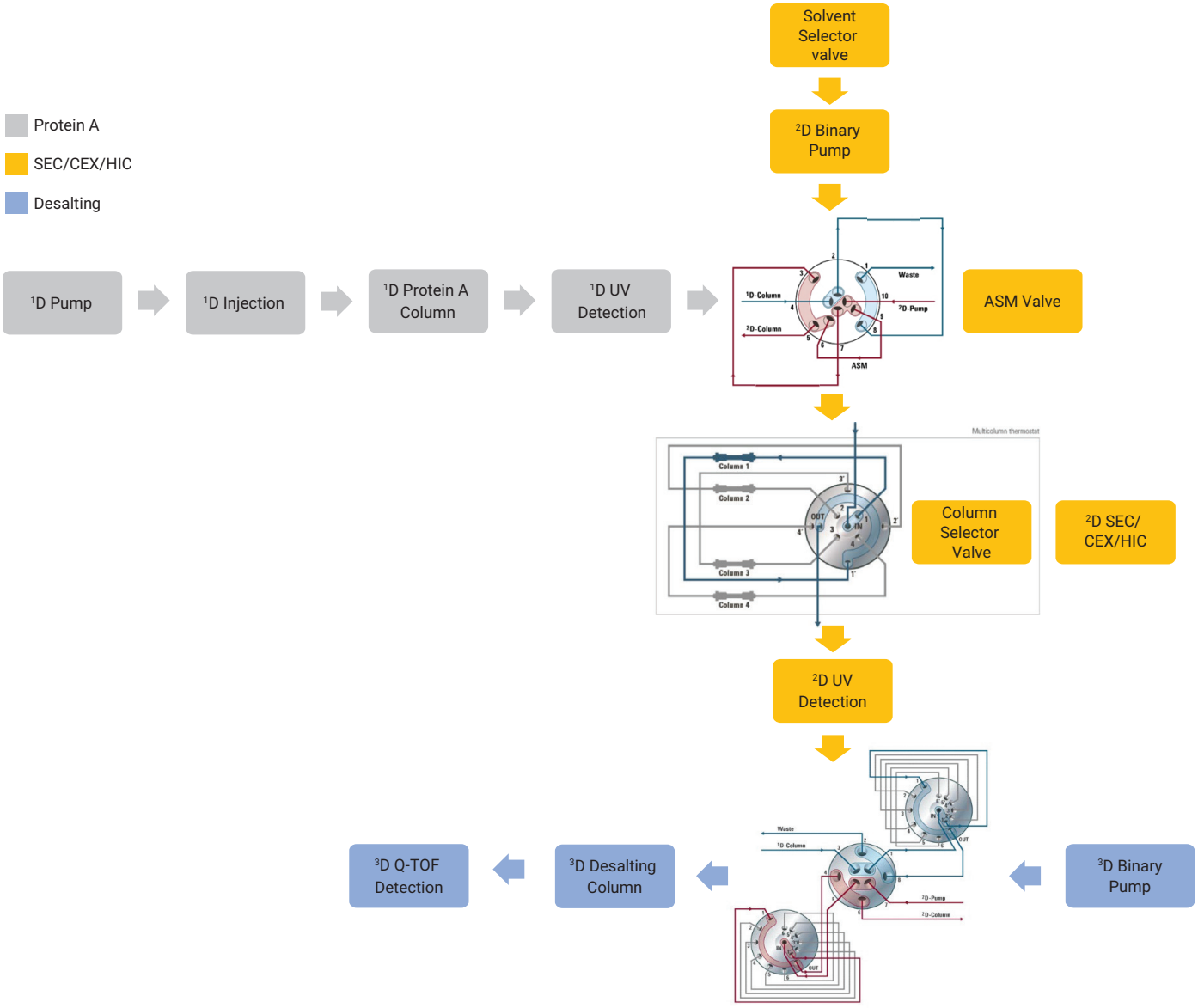

Figure 1. 3D-LC/MS configuration incorporating ^{1}D Protein A separation, ^{2}D SEC/CEX/HIC, and ^{3}D SEC-MS-based desalting.

^{2}D: Selection between SEC, CEX, and HIC

- Agilent 1290 Infinity II high-speed pump (G7120A) programmed in a pump cluster with Agilent InfinityLab quick change 12-position/13-port solvent selector, 200 bar valve head (G4235A, part number 5067-4159) installed on an Agilent 1290 Infinity valve drive (G1170A)
- Agilent 1290 Infinity II multicolumn thermostat (MCT) with valve drive installed (G7116B, option 058) equipped with an Agilent InfinityLab quick change 4-column selector valve, 800 bar (G4237A, part number 5067-4279)
- Agilent 1290 Infinity II diode array detector (G7117B) with a 10 mm Agilent InfinityLab Max-Light cartridge cell (G4212-60008)

Multiple heart-cutting

- Agilent 1290 Infinity valve drive (G1170A) with 2D-LC Valve (G4236A)
- Two Agilent 1290 Infinity valve drives (G1170A) with multiple heart-cutting valves (G4242-64000) equipped with 40 µL loops

^{3}D: Desalting SEC-MS

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multicolumn thermostat (MCT) with valve drive installed (G7116B, option 058)
- Agilent 6530 LC/Q-TOF with Jet Stream ESI source (G6530A)

Note: Protein A and SEC column used for desalting are both contained in different zones of one single column compartment.

Software

- Agilent OpenLab CDS ChemStation revision C.01.07 SR4 [505]
- 2D-LC add-on software revision A.01.04 [017]
- Agilent MassHunter for instrument control (B.09.00)
- Agilent MassHunter with BioConfirm add-on for data analysis (B.07.00)

Method

^{2}D and ^{3}D were configured in the 2D-LC software, while ^{1}D settings were controlled in a regular method setup.

MassHunter Acquisition was triggered by a remote start from the 2D-LC system. Different heart-cuts were taken across the SEC, CEX, or HIC analysis, of which the first heart-cut was a blank cut that enabled preconditioning of all dimensions prior to the analysis of the heart cuts of interest. To switch between the different applied chromatographic modes in the second dimension, it is necessary to precondition the system in terms of mobile phase and flow before switching to the next mode.

^{1}D Protein A Chromatography		
Column	Agilent Bio-Monolith protein A column, 5.2 × 5 mm (p/n 5069-3639)	
Temperature	25 °C	
Mobile Phase A	50 mM sodium phosphate pH 7.45	
Mobile Phase B	500 mM acetic acid pH 2.5	
Flow Rate	0.75 mL/min	
Gradient	Time (min) %B 0.0 0 0.2 0 0.3 100 1.4 100 2.0 0 4.0 0	
Injection	0.4 to 40 µg (calibration curve Protein A) 40 µg (CEX and main SEC) 100 µg (HIC) 500 µg (HMW SEC)	
DAD Detection	280 nm	
Peak Width	>0.05 min (5 Hz)	
ASM	^{2}D CEX and HIC	^{2}D SEC
	0 min: Pos 2 (Port 1 → 8 ASM) 1.7 min: Pos 4 (Port 1 → 3 ASM)	0 min: Pos 1 (Port 1 → 8) 1.7 min: Pos 5 (Port 1 → 3)
	^{1}D Protein A peak was collected in an 80 µL loop installed on the ASM valve prior to transfer to the ^{2}D chromatographic mode.	

^{2}D Size-Exclusion Chromatography		
Column	Agilent AdvanceBio SEC 300A, 7.8 × 300 mm, 2.7 µm (p/n PL1180-5301)	
Temperature	25 °C	
Mobile Phase	150 mM sodium phosphate pH 7.0	
Flow Rate	0.7 mL/min	
Run Time	30 min	
DAD Detection	214 and 280 nm	
Peak Width	>0.05 min (5 Hz)	
Column Selection Valve	Pos 2 (Port 2 → 2')	
MHC	Originator	Clone
	Blank: 4.94 min	Blank: 4.94 min
	HMW: 9.62 min	HMW: 9.36 min
	Main: 10.8 min	Main: 10.8 min
	^{2}D SEC peaks were collected in 40 µL loops installed on the multiple heart-cutting valve prior to transfer to ^{3}D desalting SEC-MS.	

^{2}D Cation-Exchange Chromatography		
Column	Agilent Bio MAb NP5 PK, 2.1 × 250 mm, 5 µm (p/n 5190-2411)	
Temperature	25 °C	
Mobile Phase A	25 mM sodium phosphate pH 7.0	
Mobile Phase B	25 mM sodium phosphate pH 7.0 + 100 mM NaCl	
Flow Rate	0.2 mL/min	
Gradient	Time (min) %B 0.0 5 3.0 5 43.0 65 44.0 95 49.0 95 50.0 5 65.0 5	
DAD Detection	280 nm	
Peak Width	>0.05 min (5 Hz)	
Column Selection Valve	Pos 1 (Port 1 → 1')	
MHC	Originator	Clone
	Blank: 4.8 min	Blank: 4.8 min
	Pre 1: 13.35 min	Pre 3: 16.29 min
	Pre 2: 15.60 min	Main: 19.45 min
	Pre 3: 16.66 min	Post 1: 20.83 min
	Main: 19.89 min	Post 2: 21.61 min
	Post 1: 21.7 min	Post 3': 24.96 min
	Post 2: 22.85 min	
	Post 3: 24.01 min	
	^{2}D CEX peaks were collected in 40 µL loops installed on the multiple heart-cutting valve prior to transfer to ^{3}D desalting SEC-MS.	

^{2}D Hydrophobic Interaction Chromatography		
Column	Agilent AdvanceBio HIC, 4.6 × 100 mm, 3.5 µm (p/n 685975-908)	
Temperature	25 °C	
Mobile Phase A	1.5 M ammonium sulfate, 50 mM sodium phosphate pH 7.0	
Mobile Phase B	50 mM sodium phosphate pH 7.0/IPA (80/20) (v/v)	
Flow Rate	0.5 mL/min	
Gradient	Time (min) %B 0.0 0 2.0 0 8.0 30 38.0 45 39.0 100 43.0 100 44.0 0 50.0 0	
DAD Detection	280 nm	
Peak Width	>0.05 min (5 Hz)	
Column Selection Valve	Pos 3 (Port 3 → 3')	
MHC	Originator	Clone
	Blank: 9.92 min	Blank: 9.92 min
	Pre: 20.54 min	Pre: 20.54 min
	Main: 22.42 min	Main: 22.42 min
	Post: 24.26 min	Post: 24.26 min
	^{2}D HIC peaks were collected in 40 µL loops installed on the multiple heart-cutting valve prior to transfer to ^{3}D desalting SEC-MS.	

^{2}D Preconditioning Run		
Mobile Phase	Initial conditions ^{2}D chromatographic mode	
Gradient	Time (min) Flow rate 0.0 0 0.1 0 1.0 1.0 11.0 1.0 11.01 0.1 11.5 0.1 12.5 Flow rate initial conditions ^{2}D mode 15.0 Flow rate initial conditions ^{2}D mode	
ASM	**^{2}D SEC conditioning:** One conditioning run with H_2O is performed for flushing the ASM capillaries prior to a conditioning run with initial conditions and ASM function disabled. **^{2}D CEX and HIC conditioning:** One conditioning run with start mobile phase composition.	
	^{2}D SEC	Pos 2 (Port 1 → 8 ASM) – flush with H_2O Pos 1 (Port 1 → 8) – SEC conditioning
	^{2}D CEX	Pos 2 (Port 1 → 8 ASM)
	^{2}D HIC	Pos 2 (Port 1 → 8 ASM)
Column Selection Valve	Time (min) Flow rate 0.00 Use current position 0.05 Pos 4 (Port 4 → 4') 11.25 Valve position initial conditions ^{2}D mode	
During the conditioning run, ^{1}D and ^{3}D initial conditions are applied.		

MS data processing

Measured signals were deconvoluted using the BioConfirm Maximum Entropy deconvolution algorithm incorporated in the MassHunter software.

Results and discussion

A scheme of the 3D-LC/MS multi-attribute analyzer incorporating ^{1}D Protein A chromatography, ^{2}D SEC, CEX, or HIC and ^{3}D SEC-MS based desalting is shown in Figure 1.

The starting point is the purification of the mAb from cell culture supernatants and the determination of the mAb titer using Protein A affinity chromatography. Protein A from *Staphylococcus aureus* has a strong affinity for the Fc domain of IgG, allowing its separation from matrix components present in cell culture supernatants. Following binding at neutral pH, the mAb is eluted using a one-step gradient towards an acidic mobile phase. Quantitation of the mAb (titer determination) occurs by integration of the eluting peak at UV 280 nm.

The Protein A peak is subsequently collected in the 80 µL loop installed on the ASM valve and transferred to the second dimension by a valve switch at a predefined time (not controlled by the 2D-LC software but programmed in the general method settings). The chromatographic mode used in the second dimension (SEC, CEX, or HIC) is determined by the column selector valve. When selecting CEX or HIC, the ASM function is activated, making the ^{1}D eluent composition compatible with the ^{2}D. This function is disabled for SEC as the dilution effect inherent to ASM causes peak broadening. Peaks eluting from the ^{2}D column are detected by the second DAD, enabling determination of the level of acidic and basic variants (CEX), high (HMW), and low molecular weight (LMW) variants (SEC) or hydrophobic variants (HIC).

^{3}D Size Exclusion Chromatography		
Column	Agilent AdvanceBio SEC 300A, 4.6 × 50 mm, 2.7 µm (p/n PL1580-1301)	
Temperature	25 °C	
Mobile Phase A	0.1% FA in 500 mM ammonium formate	
Mobile Phase B	0.1% FA in 20% ACN (v/v)	
Flow Rate	0.4 mL/min (0.8 mL/min after ^{2}D HIC)	
Gradient	Time (min) %B 0.0 100 3.0 100 3.01 0 8.0 (15.0 after ^{2}D HIC) 0 8.01 (15.01 after ^{2}D HIC) 100 20.0 (30.0 after ^{2}D HIC) 100	
MS Detection		
Diverter Valve	**^{2}D SEC and CEX**	**^{2}D HIC**
	0 min: Flow to MS 1.55 min: Flow to Waste	0 min: Flow to MS 0.75 min: Flow to Waste
Source		
	Positive ionization	
Drying Gas Temperature	300 °C	
Drying Gas Flow	8 L/min	
Nebulizer Pressure	35 psi	
Sheath Gas Temperature	350 °C	
Sheath Gas Flow	11 L/min	
Capillary Voltage	3,500 V	
Nozzle Voltage	1,000 V	
Fragmentor	350 V	
Acquisition		
Mode	High mass range (1 GHz)	
Data Acquisition Range	*m/z* 500 to 10,000	
	1 spectrum/s	
	Profile acquisition	

^{2}D peaks are collected in the 40 µL loops installed on a multiple heart-cutting valve and transferred one by one to the ^{3}D SEC-MS. The ^{3}D SEC column serves as a desalting tool to separate the protein from the nonvolatile salts used in the elution buffers (SEC/CEX/HIC), which is indispensable for the subsequent MS measurement. The buffer excipients are retained on the column while mAb-related analytes elute first into the MS system. The diverter valve, configured as a repetitive event and programmed in the MassHunter software, is switched to waste before elution of the buffer excipients.

The performance of this 3D-LC multi-attribute analyzer was evaluated with commercial trastuzumab (Herceptin) and trastuzumab-producing CHO clones originating from a biosimilar development campaign. Trastuzumab is a humanized IgG1 binding the HER2 receptor and finding use in the treatment of HER2 positive metastatic breast cancer.

Chromatograms and spectra obtained for trastuzumab originator and CHO clone 10 are shown in Figure 2. High quality and informative [1]D Protein A and [2]D SEC, CEX, and HIC chromatograms are obtained with an overall higher purity observed for trastuzumab originator compared to trastuzumab derived from CHO clone 10. The MS measurement of the main peak of trastuzumab originator (indicated in blue) of each chromatographic mode shows comparable deconvoluted MS spectra and glycoform distribution. However, a high amount of adduct formation is observed in the HIC run, which is caused by the extensive amount of ammonium sulfate present in the mobile phase.

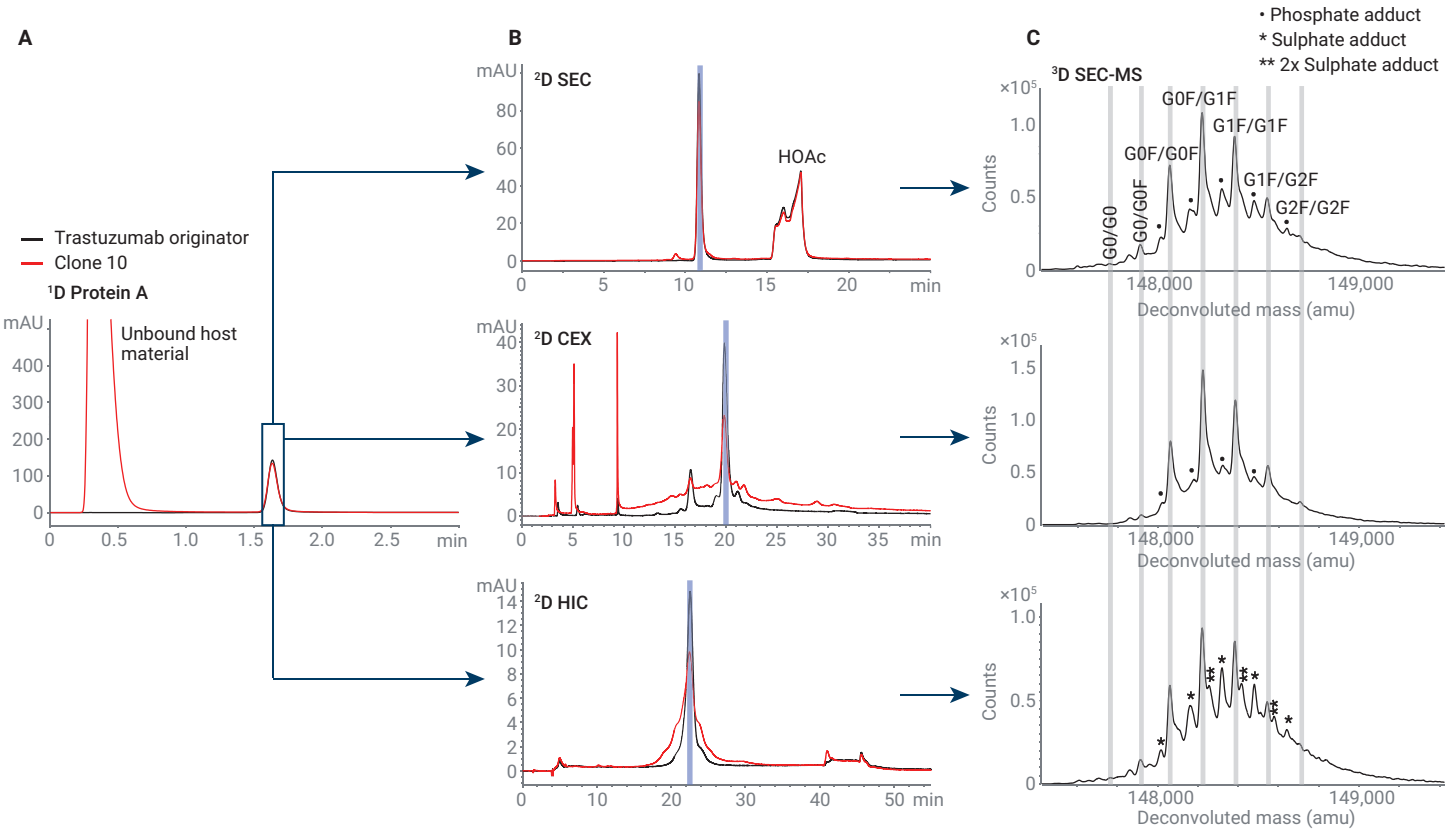

Figure 2. (A) [1]D Protein A chromatogram of the trastuzumab originator and CHO clone 10 supernatants. (B) [2]D SEC, CEX, and HIC chromatograms of Protein A peak at 1.6 minutes. (C) Deconvoluted [3]D SEC-MS spectra of trastuzumab originator main [2]D SEC, CEX, and HIC peak. [2]D heart cuts taken are indicated in blue.

[1]D Protein A titer determination

A calibration curve was composed by injecting 20 µL of commercial trastuzumab at a concentration varying from 0.02 to 2.0 µg/µL. Thereafter, 20 µL of the CHO clone supernatants was injected and titer determination was performed by using the calibration curve. Protein A chromatograms and calibration curve are shown in Figure 3 (A, B, and C). The host material is eluting in the flowthrough, generating a highly intense signal, while the mAb is retained on the Protein A column and is only released after lowering the pH (Figure 3B). It does not come as a surprise that this flowthrough signal is absent in the originator, as this represents the marketed and highly purified product. From the Protein A chromatograms, absolute mAb concentrations can be determined by linking the peak area to the external calibration curve constructed from the dilution series of trastuzumab (Figure 3C). Titers of 0.40, 0.09, 0.57, 0.74, and 1.0 µg/µL are obtained for, respectively, clone 3, 6, 8, 9, and 10. From these findings, a distinction can be made between low- and high-producing clones. Thereafter, the Protein A peak was collected and transferred to [2]D SEC, CEX, and HIC and chromatograms are shown in Figure 3 (D, E, and F).

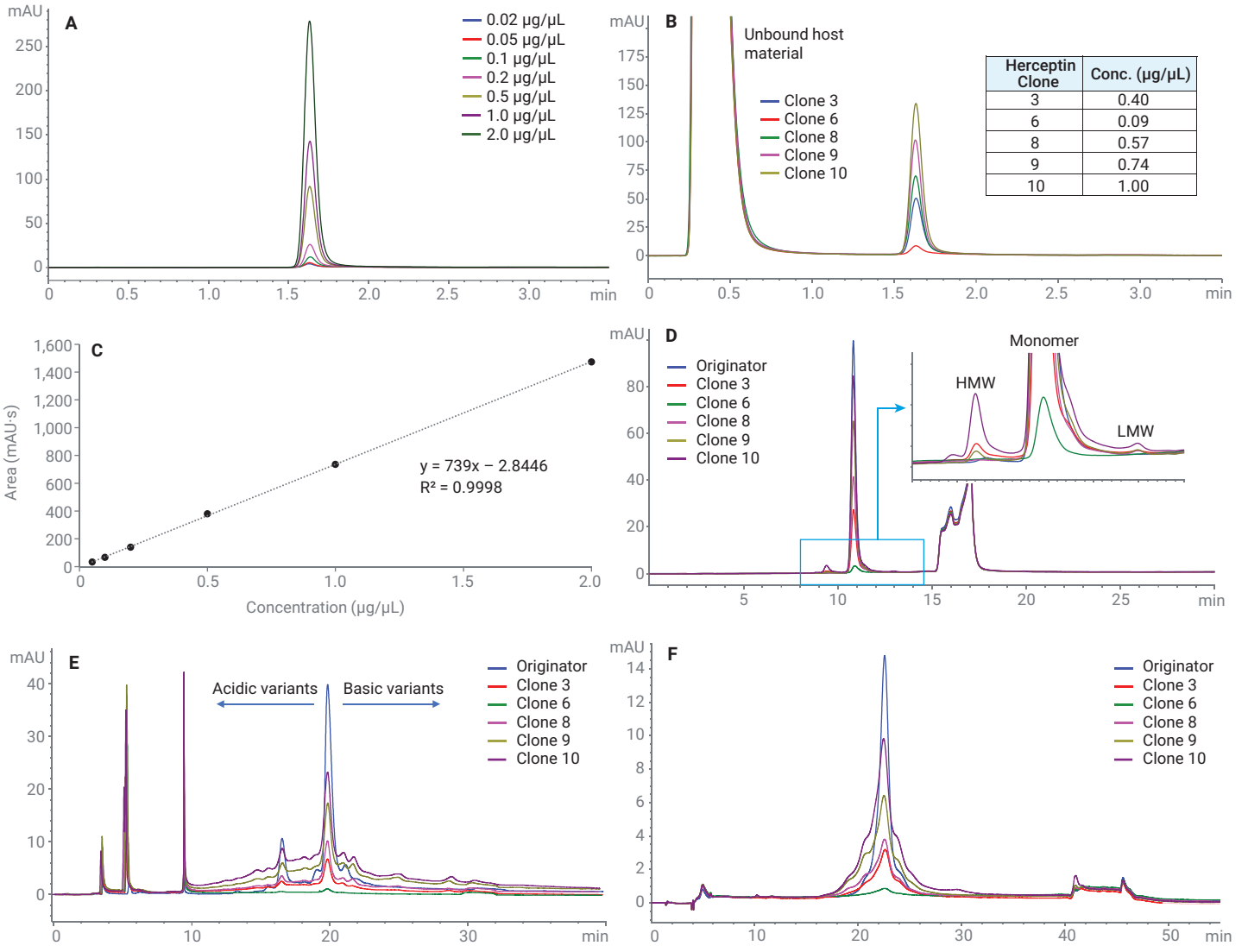

Figure 3. Heart-cutting Protein A–SEC/CEX/HIC analysis of trastuzumab originator and five trastuzumab-producing CHO clones. (A) [1]D Protein A chromatograms at UV 280 nm of the dilution series of trastuzumab originator (0.02, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 µg/µL). (B) [1]D Protein A chromatograms at UV 280 nm of five trastuzumab-producing CHO clones. (C) Calibration curve based on [1]D UV 280 nm peak areas. (D) [2]D SEC chromatograms at UV 280 nm of trastuzumab originator and five trastuzumab-producing CHO clones and zoomed view showing the detection of HMW and LMW variants. (E) [2]D CEX chromatograms at UV 280 nm of trastuzumab originator and five trastuzumab-producing CHO clones. (F) [2]D HIC chromatograms at UV 280 nm of trastuzumab originator and five trastuzumab-producing CHO clones.

The CHO clones mainly differ in intensity of the eluting variants, which is directly linked to the titer.

3D-LC/MS with ^{2}D SEC option

Purity in terms of high and low molecular weight variants (HMW and LMW) can be assessed by transferring the Protein A peak to ^{2}D SEC. SEC chromatograms of trastuzumab originator and five trastuzumab-producing CHO clones are shown in Figures 4A and 4B.

Differences in HMW and LMW variants are observed between the different samples. The CHO clones contain more HMW and LMW species than observed in trastuzumab originator. In addition, HMW variants of the CHO clones are eluting earlier than the HMW variant observed in the originator, indicating that there is a difference in the nature of the HMWs. Note that in the ^{2}D SEC chromatogram, a very broad peak is observed at 15 minutes corresponding to the Protein A elution buffer (acetic acid, HOAc). Furthermore, injecting the Protein A-purified mAb in an acetic acid plug increases the tailing factor on the main peak compared to a one-dimensional SEC analysis from a buffer at neutral pH.

The ^{3}D SEC-MS raw and deconvoluted spectra of trastuzumab originator and CHO clone 10 are shown in Figures 4C to 4F. Note that, due to the high flow rate applied, only a small part of the ^{2}D SEC peak is collected and transferred to the ^{3}D desalting SEC-MS, causing low intensity MS spectra for less abundant peaks. Consequently, a higher ^{1}D Protein A load of 500 µg was applied to have characterization data for the HMW peaks. Alternatively, the multiple heart-cutting loop size could be increased, but this would deteriorate SEC separation.

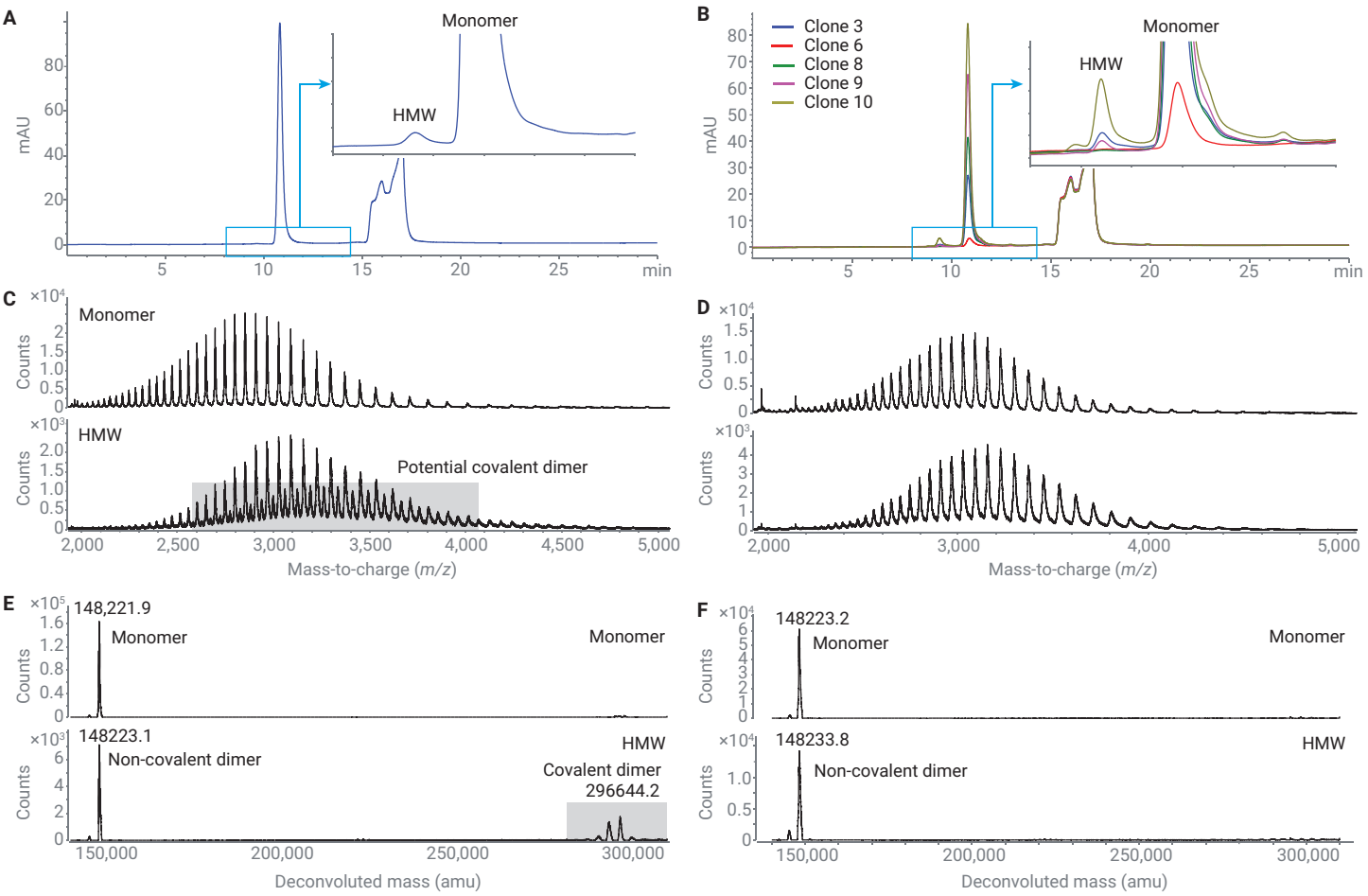

Figure 4. 3D-LC/MS analysis of trastuzumab originator and five trastuzumab-producing CHO clones using the ^{2}D SEC option. (A) ^{2}D SEC chromatogram at UV 280 nm of trastuzumab originator. (B) ^{2}D SEC chromatograms at UV 280 nm of five trastuzumab-producing CHO clones. (C) ^{3}D raw SEC-MS spectra of trastuzumab originator main and HMW peak. (D) ^{3}D raw SEC-MS spectra of CHO clone 10 main and HMW peak. (E) ^{3}D deconvoluted SEC-MS spectra of trastuzumab originator main and HMW peak. (F) ^{3}D deconvoluted SEC-MS spectra of CHO clone 10 main and HMW peak.

While the raw monomer spectra are similar, the raw HMW spectra differ between trastuzumab originator and CHO clone 10. A shift of the entire charged envelope to higher *m/z* values and the presence of lower abundant intermediate *m/z* peaks suggest a mix of covalent and noncovalent dimers in the case of trastuzumab originator. In the case of trastuzumab-producing CHO clone 10, only a shift of the charged envelope is observed, suggesting the presence of noncovalent dimers. The latter dimers do not survive the denaturing ^{3}D SEC-MS conditions and will hence fall apart in monomers. Indeed, when deconvoluting the data, an MW corresponding to dimer is only observed in the HMW peak of trastuzumab originator. For CHO clone 10 HMW, the same MW is obtained as the monomer, since noncovalent bonds are broken due to the denaturing conditions used.

3D-LC/MS with ^{2}D CEX option

CEX is an excellent tool to highlight charge variants that might arise from modifications such as deamidation, lysine truncation, and N-terminal cyclization. ^{2}D CEX chromatograms of trastuzumab originator and five trastuzumab-producing CHO clones are shown in Figures 5A and 5B. Overall, ^{2}D CEX chromatograms are comparable with a higher purity revealed for the trastuzumab originator. An intense peak is observed at 3, 5, and 10 minutes in the chromatogram of the CHO clones probably caused by the acidic plug originating from the Protein A elution conditions.

The deconvoluted ^{3}D SEC-MS spectra of selected charge variants for trastuzumab originator and CHO clone 10 are shown in Figure 5C. It must be noted that the load was increased to 100 µg for the low abundant peaks (peaks 1 and 5) in order to have more intense MS spectra. Trastuzumab originator and CHO clone 10 have comparable MW values and glycosylation patterns for the main ^{2}D CEX peak (peaks 3 and 6). ^{2}D CEX peaks 1, 2, and 4 have similar MW values to the main peak (within the mass accuracy of the instrument) pointing in the direction of deamidation or isomerization events. Harris *et al.*[9] and Verscheure *et al.*[10], who performed CEX fraction collection and peptide mapping in, respectively, an offline and online manner, confirmed that peak 1 corresponds to a double-deamidated variant, peak 2

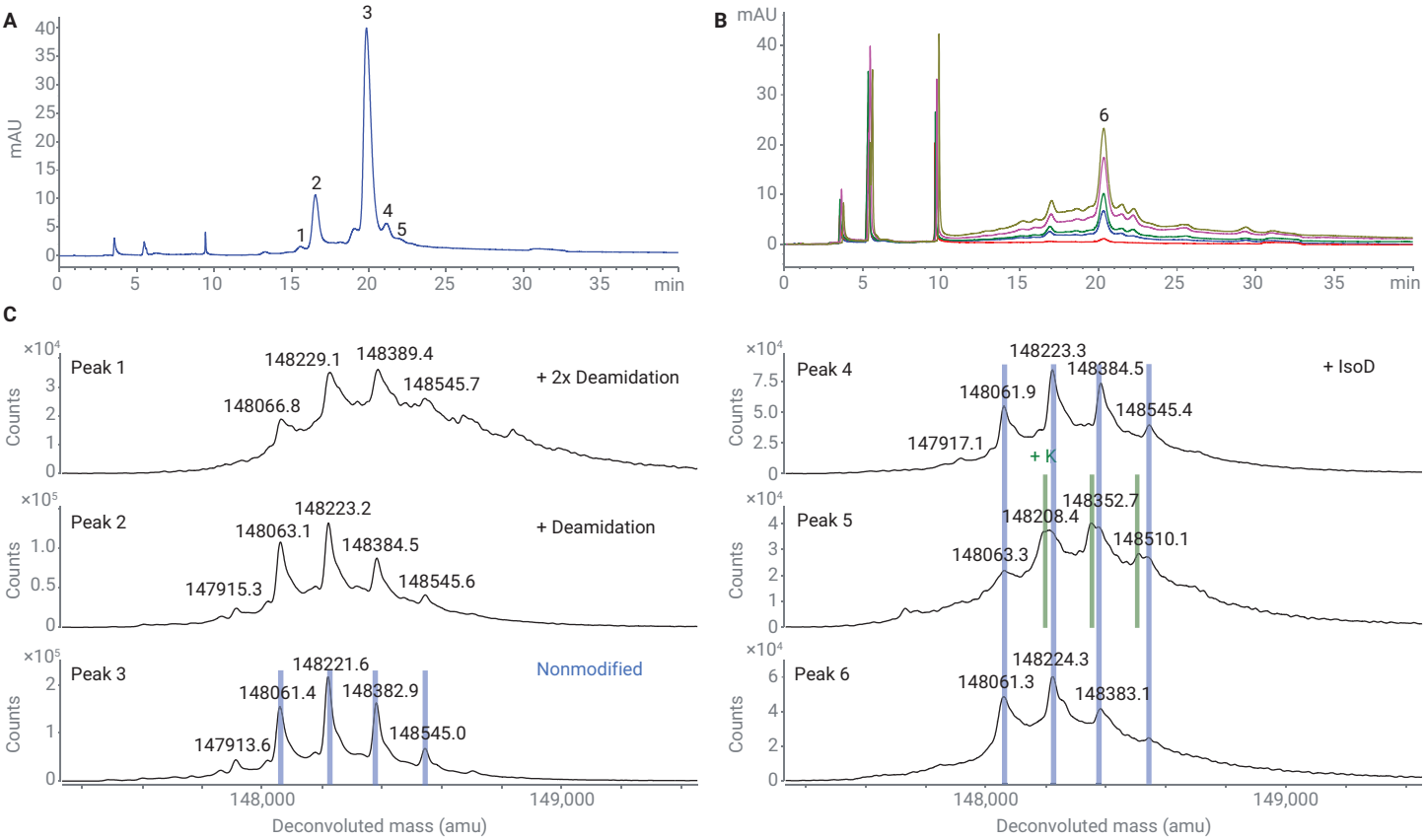

Figure 5. 3D-LC/MS analysis of trastuzumab originator and five trastuzumab-producing CHO clones using the ^{2}D CEX option. (A) ^{2}D CEX chromatogram at UV 280 nm of trastuzumab originator. (B) ^{2}D CEX chromatograms at UV 280 nm of five trastuzumab-producing CHO clones. (C) ^{3}D deconvoluted SEC-MS spectra of trastuzumab originator and CHO clone 10 collected peaks.

to a single-deamidated variant, and peak 4 to an isoaspartate-containing variant. In ^{2}D CEX peak 5, a variant with a mass increase of approximately 128 Da is observed, corresponding to a C-terminal lysine.

3D-LC/MS with ^{2}D HIC option

HIC is known as a workhorse in downstream processing of protein biopharmaceuticals and this technique also has added value as an analytical tool. The separation mechanism is based on the adsorption of the protein on a weak hydrophobic stationary phase in the presence of a high salt concentration. By decreasing the salt concentration, proteins desorb in order of increasing hydrophobicity, and antibody heterogeneities such as oxidation, deamidation, isomerization, C-terminal lysine, N-terminal cyclization, and clipping can be highlighted.

HIC chromatograms of trastuzumab originator and five trastuzumab-producing CHO clones are shown in Figures 6A and 6B.

Deconvoluted MS spectra of the main peak for trastuzumab originator and trastuzumab-producing CHO clone 10 are shown in Figures 6C and 6D, respectively. The load was increased to 100 µg to obtain qualitative MS spectra. Despite the ^{3}D SEC desalting, convoluted spectra were obtained due to adduct formation resulting in substantially decreased sensitivity. Further method development is necessary to find a suitable buffer substitute for ammonium sulfate/phosphate buffer.

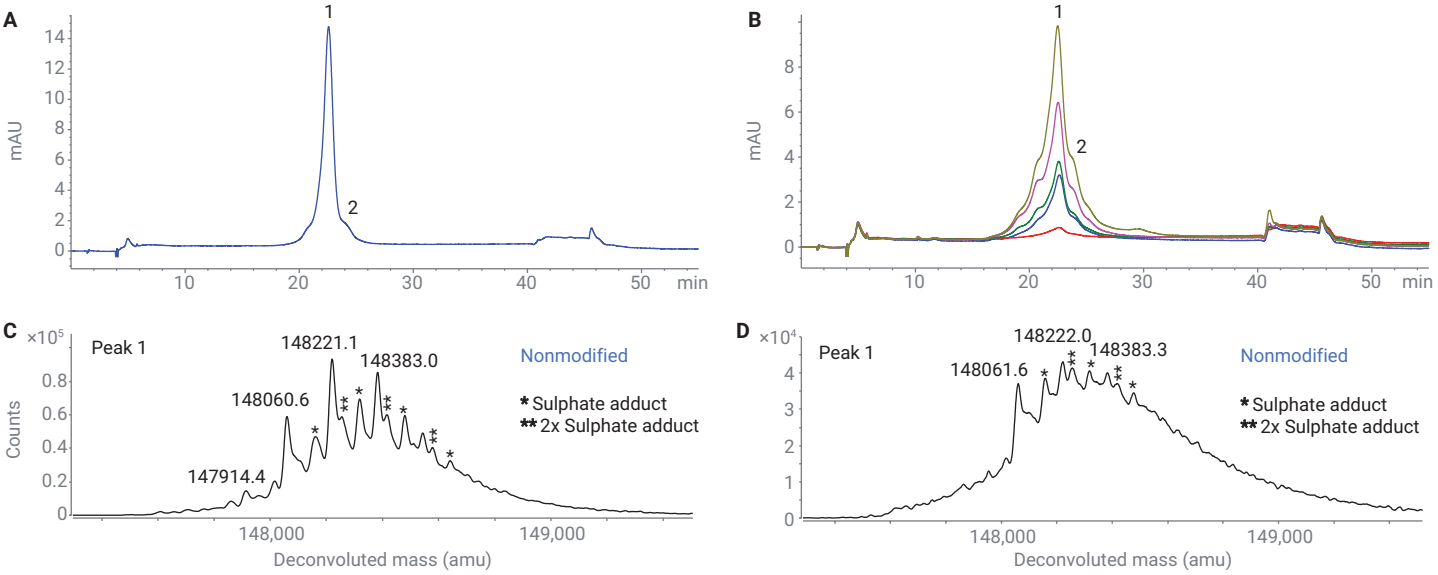

Figure 6. 3D-LC/MS analysis of trastuzumab originator and five trastuzumab-producing CHO clones using the ^{2}D HIC option. (A) ^{2}D HIC chromatogram at UV 280 nm of trastuzumab originator. (B) ^{2}D HIC chromatograms at UV 280 nm of five trastuzumab-producing CHO clones. (C) ^{3}D deconvoluted SEC-MS spectrum of trastuzumab originator collected peak. (D) ^{3}D deconvoluted SEC-MS spectrum of CHO clone 10 collected peak.

Automated ^{2}D mode switching

The above-described 3D-LC/MS multi-attribute analyzer offers the option to choose between three different chromatographic modes (SEC, CEX, or HIC) in the second dimension by the incorporation of a column selector valve. As the different mobile phase compositions are not compatible for all chromatographic modes, evaluation of automated switching between ^{2}D modes was required. To avoid the presence of noncompatible mobile phases on a certain column, the system is preconditioned by flushing with the mobile phase composition of the next desired chromatographic mode, using the bypass position of the column selector valve, and following with by a blank run to fully condition the column before injection of the sample.

Evaluation was performed by running a sequence where each chromatographic mode was run sequentially. For example, after a ^{2}D SEC run, the system was preconditioned to the CEX conditions and trastuzumab was injected after the blank run. This chromatogram was compared to the chromatogram obtained after a ^{2}D CEX run and ^{2}D HIC run and shown in the first column in Figure 7. Overall, comparable chromatograms were obtained for all chromatographic modes. Therefore, it can be concluded that different ^{2}D modes can be run in one sequence without losing chromatographic quality, which is of course the ultimate goal of the setup.

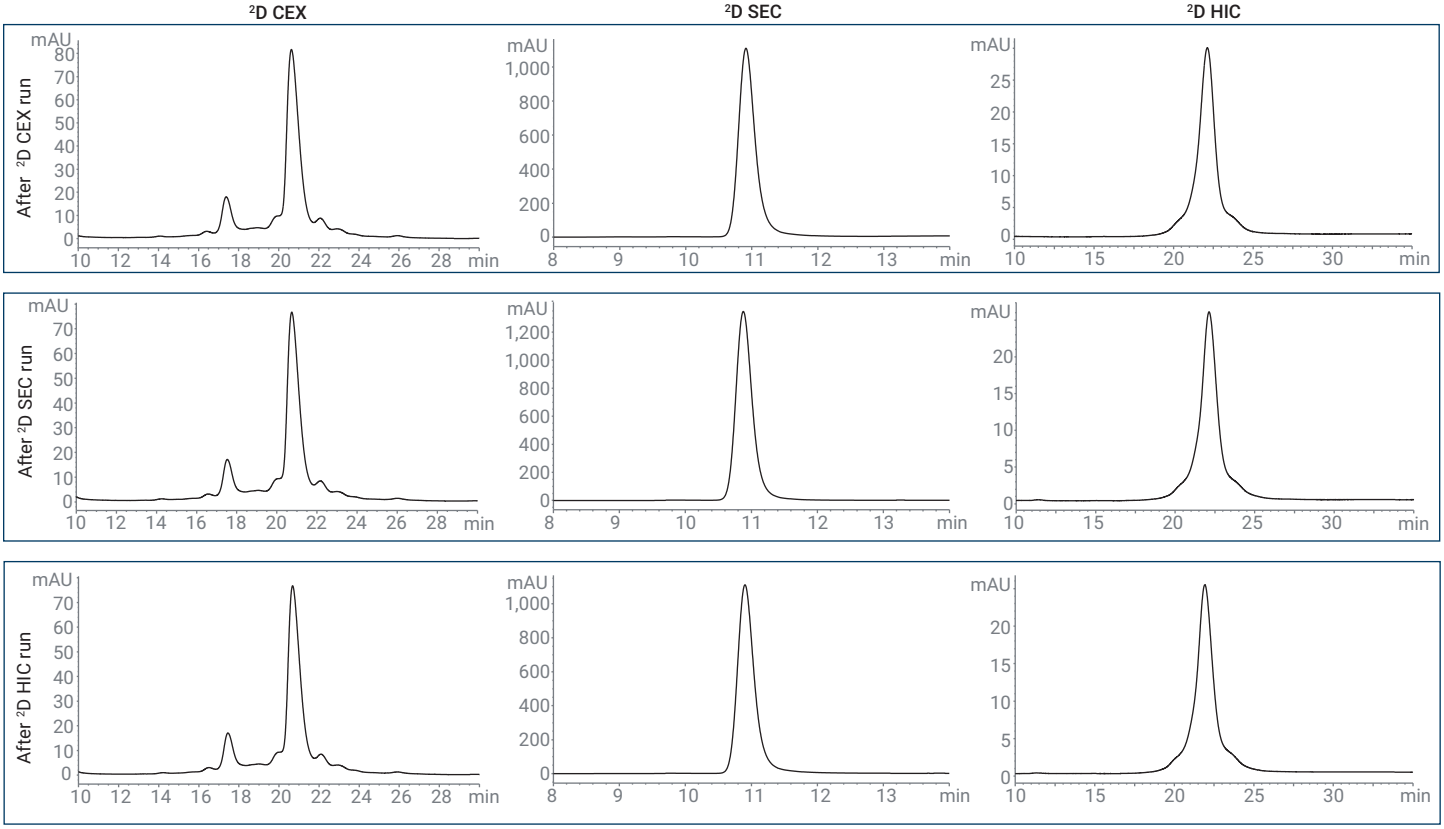

Figure 7. ^{2}D UV 280 nm chromatograms obtained during evaluation of the ^{2}D mode switching between SEC, CEX, and HIC.

Conclusion

A fully automated 3D-LC/MS multi-attribute analyzer incorporating Protein A affinity chromatography in the first dimension with a multimethod option (choice between SEC, CEX, or HIC) in the second dimension and desalting SEC-MS in the third dimension was described. This setup enables purification of mAbs from cell culture supernatants and determination of mAb titer, size/charge/hydrophobic variants, molecular weight, amino acid sequence, and post translational modifications. The setup can automatically switch between the different ^{2}D methods without impacting resolution.

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