Application Note

Pharma & Biopharma

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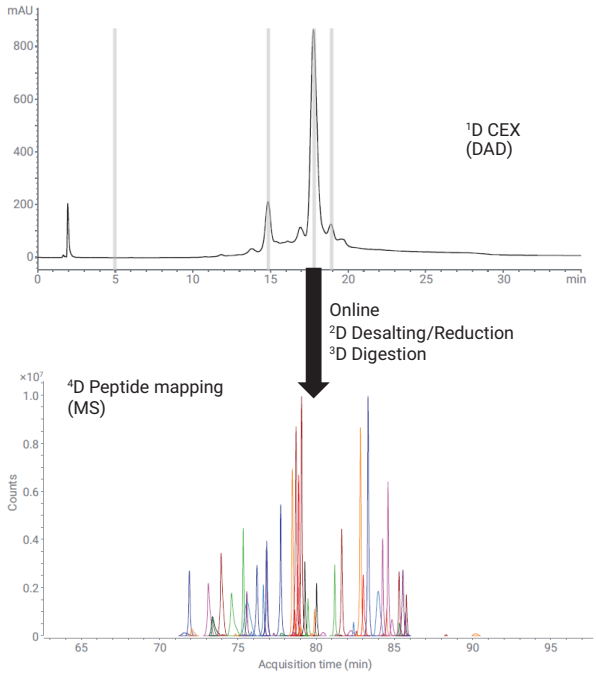
Fully Automated Characterization of Monoclonal Antibody Charge Variants Using 4D-LC/MS



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Abstract

This application note describes the fully automated and in-depth characterization of monoclonal antibody (mAb) charge variants by four-dimensional liquid chromatography/mass spectrometry (4D-LC/MS) using the Agilent InfinityLab 2D-LC Solution and the Agilent 6545 LC/Q-TOF system. Charge variants resolved by cation-exchange chromatography (CEX) are collected in loops installed on a multiple heart-cutting valve and consecutively subjected to online desalting, denaturation, reduction, and tryptic digestion prior to LC/MS-based peptide mapping.

Introduction

Protein biopharmaceuticals have emerged as important therapeutics for the treatment of various diseases including cancer, cardiovascular diseases, diabetes, infection, inflammatory, and autoimmune disorders.[1-3] Protein biopharmaceuticals come in many flavors and include monoclonal antibodies (mAbs), antibody-drug conjugates (ADCs), fusion proteins, hormones, growth factors, cytokines, therapeutic enzymes, blood factors, vaccines, and anticoagulants. Given their obvious benefits in terms of safety and efficacy, these molecules have substantially reshaped the pharmaceutical market, and today, over 350 products have been approved for human use in the United States and the European Union.[1-3] This represents approximately one quarter of the total pharmaceutical market, with mAbs being the fastest growing class of pharmaceuticals.

Together with a huge therapeutic potential, these molecules come with an enormous, analytically demanding structural complexity.[1,2] In contrast to small molecule drugs, biopharmaceuticals are large (mAbs have an MW of approximately 150 kDa) and heterogeneous. They are the product of one or a couple of genes. However, hundreds of possible variants that differ in post-translational modifications (PTMs), amino acid sequence, higher-order structure, etc. may coexist, all making up the profile, safety, and efficacy of the product.[1-3] Consequently, their in-depth structural characterization involves a significant number of analytical tools, with chromatography (LC) and mass spectrometry (MS) at the forefront.

A key technology to study charge variants that might arise from PTMs such as asparagine deamidation, C-terminal lysine truncation, N-terminal cyclization (pyroglutamate formation), sialylation, etc. is CEX. In CEX, electrostatic interaction between the anionic groups of the stationary phase and cationic groups on the protein surface form the basis of the separation. The protein is loaded on the column at a mobile phase pH below its isoelectric point (pI), and elution is achieved using a salt or pH gradient. CEX buffers are typically composed of nonvolatile constituents, making these methods incompatible with MS. Peak identification is a laborious task involving peak collection and desalting prior to MS analysis.[4] With the recent introduction of commercial and robust 2D-LC instrumentation, this series of events is now commonly performed in an online automated manner.[5-9] Peaks eluting from the CEX column are stored in loops and subjected to online desalting using reversed-phase (RPLC) or size exclusion chromatography (SEC) prior to MS measurement. Both comprehensive (LC×LC) and (multiple) heart-cutting 2D-LC (LC-LC) have been used.[5-9] To unambiguously identify CEX peaks, however, peptide mapping is required. While protein measurement is indicative of identity and highlights dominant modifications with mass differences beyond the mass accuracy of the MS instrument, it typically does not provide the actual amino acid sequence, nor does it allow us to localize modifications. Addressing the latter, and inspired by previous work,[10-12] the current application note describes a fully automated online 4D-LC/MS setup incorporating first dimension (^{1}D) CEX, peak collection, ^{2}D desalting, denaturation, reduction, ^{3}D trypsin digestion, and ^{4}D RPLC/MS-based peptide mapping for the in-depth characterization of mAb charge variants.

Experimental

Materials

Acetonitrile (HPLC-S), water (ULC/MS), and formic acid (ULC/MS) were obtained from Biosolve (Valkenswaard, The Netherlands). NaH_2PO_4, $Na_2HPO_4 \cdot 2H_2O$, NaCl, NH_4HCO_3, Tris base, and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO, USA). UltraPure Tris-HCl pH 7.5 was purchased from ThermoFisher Scientific (Waltham, MA, 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).

Sample preparation

Trastuzumab was diluted to 7 mg/mL in ^{1}D CEX mobile phase A (MPA: 10 mM sodium phosphate pH 7.65). Deamidation was induced by incubating trastuzumab at 37 °C for 3 days in high pH conditions (100 mM Tris pH 9.0) and subsequently buffer-exchanged to 7 mg/mL in ^{1}D CEX mobile phase A.

Instrumentation

An Agilent 1290 Infinity II 2D-LC system equipped with the multiple heart-cutting option, an additional Agilent 1260 Infinity II quaternary pump and Agilent 1260 Infinity II isocratic pump, two additional 2-position/6-port valves, and a zero dead volume T-piece were used. Stainless steel tubing with an internal diameter of 0.12 mm was applied. The configuration is schematically represented in Figure 1 and summarized in this application note. Diode array detection (DAD) was used in the first (CEX) and fourth dimension (RPLC). Additionally, an Agilent 6545 LC/Q-TOF with a Jet Stream ESI source was used for detection after the fourth and final dimension.

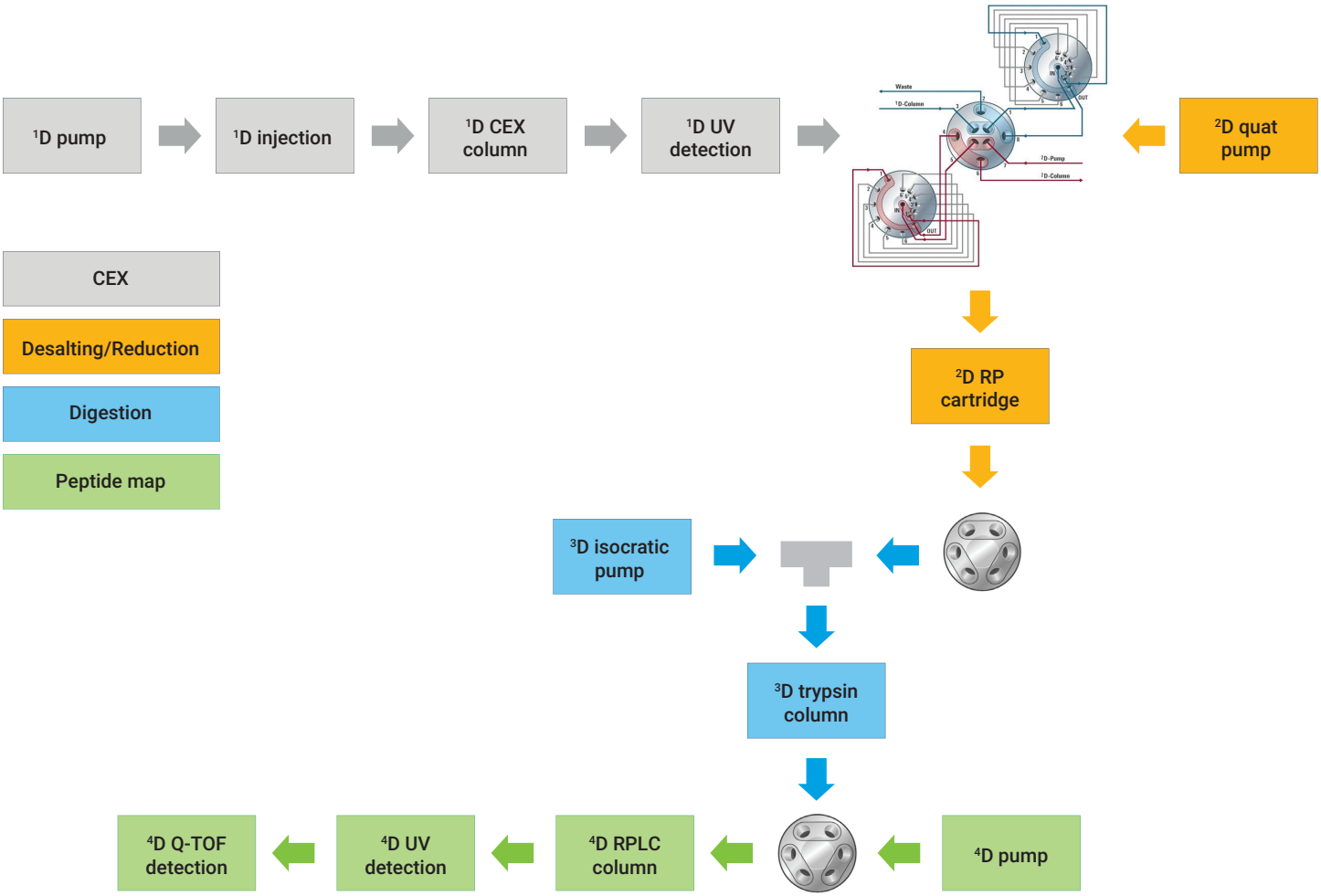

Figure 1. 4D-LC/MS configuration incorporating ^{1}D CEX separation and charge variant peak collection using multiple heart-cutting, ^{2}D RPLC-based desalting, denaturation, reduction, ^{3}D trypsin digestion, and ^{4}D RPLC-MS based peptide mapping.

Configuration

^{1}D: Cation-exchange chromatography

- G7120A Agilent 1290 Infinity II high-speed pump
- G7167B Agilent 1290 Infinity II multisampler with sample thermostat (option 101)
- G7116B Agilent 1290 Infinity II multicolumn thermostat (MCT) with valve drive installed (option 058) equipped with an Agilent InfinityLab quick change 2-position/6-port valve, 1300 bar (G4231C)
- G7117B Agilent 1290 Infinity II diode array detector with a 3.7 mm HDR InfinityLab Max-Light cartridge cell (G4212-60032)

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

2D-LC with 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

^{2}D: Reversed-phase chromatography for desalting, denaturation, and reduction

G7111B Agilent 1260 Infinity II quaternary pump with active inlet valve (AIV) (option 032)

^{3}D: Trypsin digestion

- 0100-0969 ZDV T-piece
- G7110B Agilent 1260 Infinity II isocratic pump

[4]D: Reversed-phase chromatography for peptide mapping

- G7120A Agilent 1290 Infinity II high-speed pump
- G7116B Agilent 1290 Infinity II MCT with valve drive installed (option 058) equipped with an Agilent InfinityLab quick change 2-position/6-port valve, 1300 bar (G4231C)
- G7117B Agilent 1290 Infinity II DAD with a 10 mm InfinityLab Max-Light cartridge cell (G4212-60008)
- G6545A Agilent 6545 LC/Q-TOF with Jet Stream ESI source

Note: Orachrom StyrosZyme TPCK-Trypsin and Agilent AdvanceBio peptide mapping columns are both contained in different zones of one single column compartment and maintained at 40 and 60 °C, respectively.

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

[1]D and [4]D were configured in the 2D-LC software, while [2]D and [3]D were controlled in a regular method setup and were programmed as repetitive events. The cycle time of these events was 110 minutes, identical to the [4]D cycle time programmed in the 2D-LC software. MassHunter acquisition was triggered by a remote start from the 2D-LC system.

Four heart-cuts were taken across the CEX analysis. The first heart-cut at 4.8 minutes is a blank cut, which enables preconditioning of all dimensions before the analysis of the actual CEX heart-cuts of interest.

[1]D: Cation-Exchange Chromatography			
Column	Agilent Bio MAb, nonporous (2.1 mm × 250 mm, 5 µm) (p/n 5190-2411)		
Temperature	30 °C		
Mobile phase A	10 mM sodium phosphate, pH 7.65		
Mobile phase B	10 mM sodium phosphate, pH 7.65 + 100 mM NaCl		
Flow rate	0.2 mL/min		
Gradient	**Time (min)**	**B (%)**	
	0	5	CEX analysis
	36	70	
	36.5	100	
	46	100	
	46.5	5	
	60	5	
Injection	100 µg		
Detection	220 and 280 nm		
Peak Width	> 0.025 min (10 Hz)		

Multiple Heart-Cutting [1]D > [2]D	
Sampling Timetable Trastuzumab	
Cut	**Time (min)**
1 – Blank	4.80
2 – Pre-peak	14.93
3 – Main Peak	17.55
4 – Post-peak	18.99

[2]D: Reversed-Phase Chromatography for Desalting, Denaturation, and Reduction (Manually Entered Repetitive Event)						
Column	Polymer-based desalting cartridge, 2.1 × 10 mm					
Temperature	23 °C					
Mobile Phase A	0.1% (v/v) formic acid in water					
Mobile Phase B	0.1% (v/v) formic acid in acetonitrile					
Mobile Phase C	20 mM DTT in 100 mM Tris-HCl, pH 7.5					
Gradient	**Time (min)**	**A (%)**	**B (%)**	**C (%)**	**Flow (mL/min)**	
	10	99	1	0	0.5	Desalting and focusing
	10.01	0	0	100	0.2	Reduction
	20	0	0	100	0.2	
	20.01	99	1	0	0.5	Desalting and elution
	25	99	1	0	---	
	25.01	40	60	0	---	
	27	40	60	0	0.5	
	27.01	40	60	0	0.015	Elution and digestion
	68	40	60	0	0.015	
	68.01	0	100	0	0.5	
	85	0	100	0	---	
	95	99	1	0	---	
	120	99	1	0	---	
Valve	27 min: Pos 1 → Pos 2 (start trypsin digestion) 67 min: Pos 2 → Pos 1 (start peptide mapping)					

^{3}D: Trypsin Digestion (Manually Entered Repetitive Event)			
Column	Orachrom StyrosZyme TPCK-Trypsin PEEK (2.1 × 150 mm)		
Temperature	40 °C		
Mobile Phase	50 mM NH_4HCO_3, pH 8		
Gradient	**Time (min)**	**Flow (mL/min)**	
	25	0.06	
	25.01	0.135	Digestion
	67	0.135	
	67.01	0.06	
	135	0.06	
Valve	27 min: Pos 1 -> Pos 2 (start trypsin digestion) 67 min: Pos 2 -> Pos 1 (start peptide mapping)		

Data processing

Measured signals were matched onto the trastuzumab light- and heavy-chain sequences using the BioConfirm algorithm incorporated in the MassHunter software. Mass tolerance for matching experimental data onto the sequence was set at 8 ppm. Extracted ion chromatograms (EICs) obtained at 20 ppm mass accuracy were used to monitor PTMs such as deamidation.

Results and discussion

A scheme of the fully automated online 4D-LC/MS protein analyzer, incorporating CEX, peak collection, desalting, denaturation, reduction, trypsin digestion, and peptide mapping, is shown in Figure 1. CEX peaks are collected in 40 µL loops installed on a multiple heart-cutting valve and transferred one by one to a polymeric RP cartridge where desalting, denaturation, and reduction take place. The reduced mAb, trapped on the cartridge, is subsequently eluted into the trypsin column by raising the acetonitrile concentration. Using a T-piece, trypsin digestion buffer is mixed with the reversed-phase mobile phase to have optimal digestion conditions and to reduce the acetonitrile concentration.

^{4}D: Reversed-Phase Chromatography for Peptide Mapping (Repetitive Event Controlled by 2D-LC Software)			
Column	Agilent AdvanceBio peptide mapping (2.1 × 150 mm × 2.7 µm) (p/n 651750-902)		
Temperature	60 °C		
Mobile Phase A	0.1% (v/v) formic acid in water		
Mobile Phase B	0.1% (v/v) formic acid in acetonitrile		
Flow Rate	0.4 mL/min		
Gradient	**Time (min)**	**B (%)**	
	0	1	
	8.5	1	
	9	100	
	15	100	
	16	1	
	20	1	Load digest on peptide mapping column
	64	1	
	64	1	Peptide mapping
	97	45	
	98	100	
	103	100	
	104	1	
	110	1	
DAD Detection	214 and 280 nm		
Peak Width	> 0.025 min (10 Hz)		
MS Detection			
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	8 L/min		
Capillary Voltage	3,500 V		
Nozzle Voltage	1,000 V		
Fragmentor	175 V		
Acquisition			
Mode	Extended dynamic range (2 GHz)		
Data Acquisition Range	*m/z* 100 to 3,200		
	1 spectrum/s		
	Centroid acquisition		
Switch diverter valve to MS after 67 minutes			

During the digestion, the trypsin column is in-line with the peptide mapping column, and generated peptides are focused at the head of the RPLC column. After 20 minutes, a valve switch initiates the elution of the digest into the MS.

The CEX chromatogram of the mAb trastuzumab is shown in Figure 2. Trastuzumab, commercialized as Herceptin, is a humanized IgG1 binding the HER2 receptor, thereby finding use in the treatment of HER2 positive metastatic breast cancer. With a pI of 8.45, the mAb is positively charged at the CEX mobile phase pH, thereby governing interaction with the negatively charged chromatographic resin. Upon eluting the mAb using a NaCl salt gradient, various charge variants were revealed, which were subsequently subjected to online peptide mapping. Figure 3A schematically presents the 4D-LC/MS experiment involving the analysis of three CEX peaks (pre-, main-, and post-peaks) and a CEX blank as shown in Figure 2. Figure 3B zooms in on the pressure and DAD profiles of one cycle and shows the desalting, denaturation, reduction, digestion, and peptide mapping of the main CEX peak. Over 90% sequence coverage could be obtained. Peptides identified are shown in Table 1, and an overlay of the LC/MS compound chromatograms is provided in Figure 4. Peptides not covered are typically small and/or hydrophilic and are not focused at the head of the peptide mapping column during the digestion. For this reason, they are diverted to waste.

Next to sequence information, peptide mapping also reveals modifications and modification sites. Figure 5 shows the online peptide mapping of the CEX pre-, main-, and post-peak, and in particular the EICs of two peptides (i.e., light-chain peptide ASQDVNTAVAWYQQKPGK (LC 25–42) containing a potential deamidation site at position 30, and heavy-chain peptide WGGDGFYAMDYWGQGTLVTVSSASTK (HC 99–124) containing a potential isomerization site at position 102). From the data, it could be deduced that the pre-peak corresponds to a deamidated variant, with asparagine converted to aspartate at position 30 on one of the light chains. It could also be demonstrated that the post-peak carries an isoaspartate at position 102 on one of the heavy chains. This is clearly visualized by the peak doublets corresponding to the modified and nonmodified variants. These results are in accordance with those reported by Harris *et al.*, who performed offline fraction collection and peptide mapping on Herceptin acidic and basic variants.[4]

The same experiment was performed on a high-pH stressed Herceptin sample (Figure 6). Such conditions are known to induce deamidation, thereby rendering the mAb more acidic. The CEX profile presented in Figure 6 shows an acidic shift, and the peptide map data of CEX peaks 1 and 2 show a double deamidated variant, with both light chains deamidated at position 30. CEX peaks 3 and 4 correspond to a single deamidation event, with one light chain deamidated at position 30. The difference between peaks 1 and 2, and 3 and 4 originate from another deamidation, this time at position 387 in the heavy chain. This deamidation site appears in two peptides (one fully cleaved and one miscleaved) that are apparently digested differently when a deamidation exists.

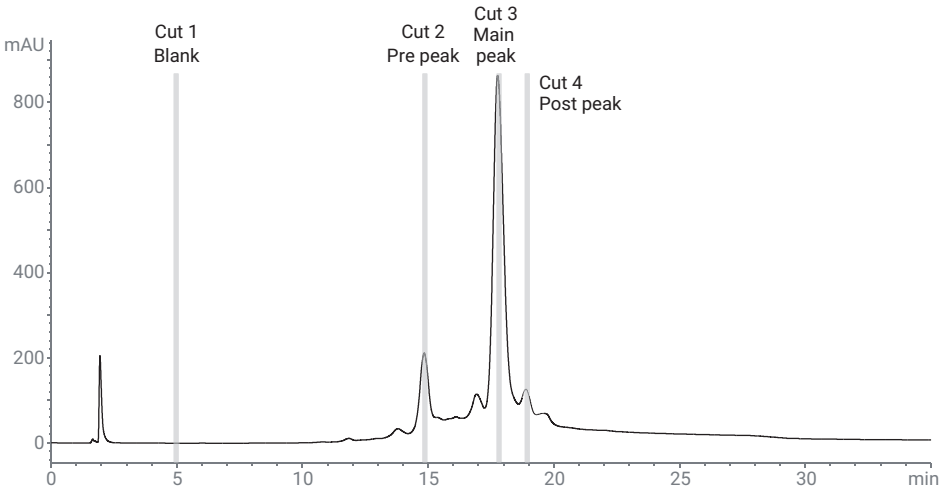

Figure 2. CEX chromatogram of the monoclonal antibody trastuzumab. Conditions according to reference 13. Heart-cuts taken are indicated in gray.

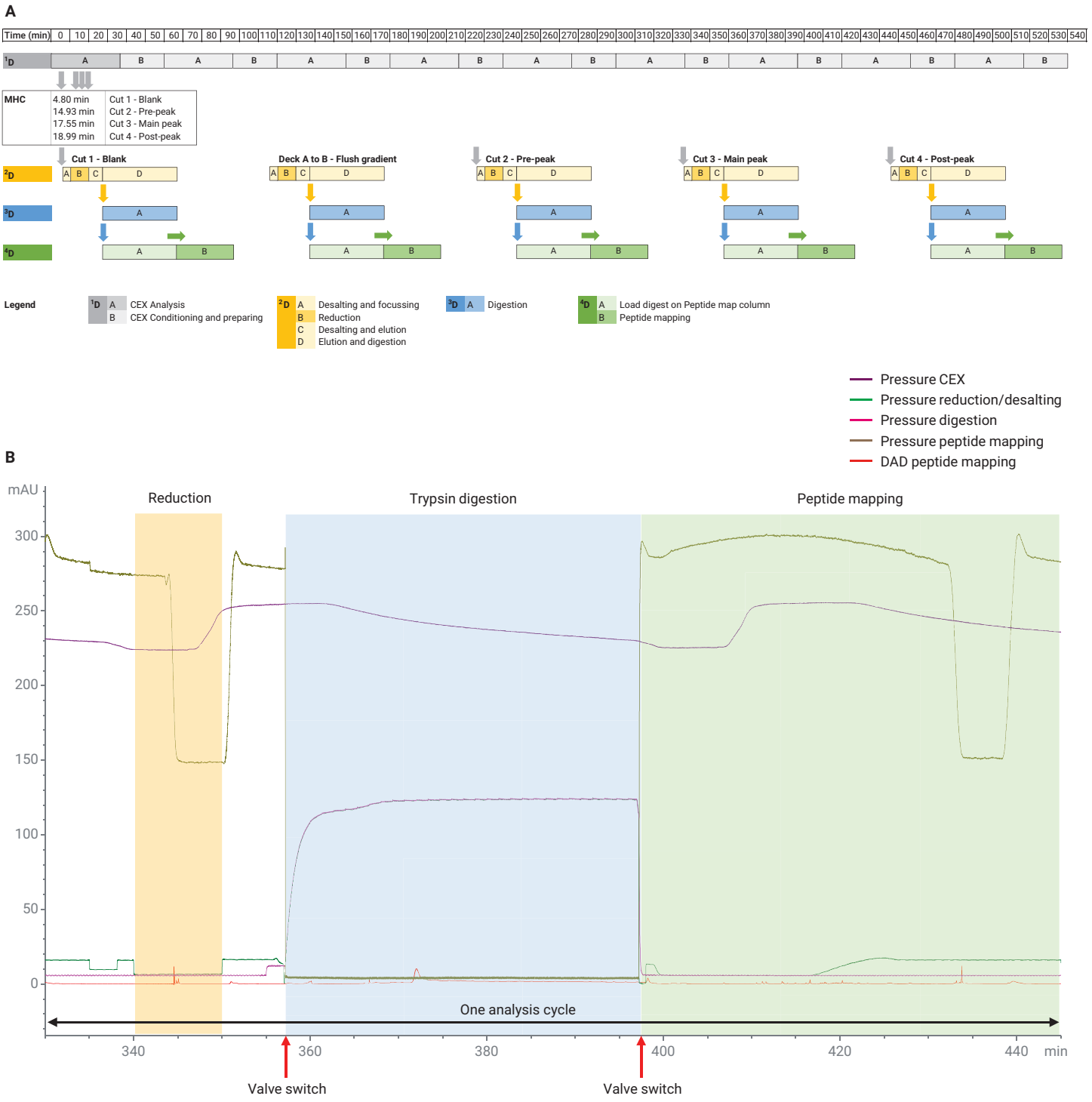

Figure 3. (A) Schematic representation of the different stages of the 4D-LC/MS experiment focusing on the pre-, main-, and post-peak as well as a CEX blank region as shown in Figure 2. (B) Focus on desalting, denaturation, reduction, digestion, and peptide mapping of the main CEX peak.

Table 1. Peptides identified in the CEX main peak following online RPLC/MS-based peptide mapping.

RT	Mass	Vol	Vol %	Sequence	Seq Loc.	Tgt. Seq. Mass	Diff. (ppm)	Missed Cleavage
79.1	1880.9972	66635668	6.61	EVQLVESGGGLVQPGGSLR	HC(001-019)	1880.9956	0.8	0
76.8	1109.5539	25727274	2.55	LSCAASGFNIK	HC(020-030)	1109.5539	0.0	0
81.2	2180.0864	16055279	1.59	LSCAASGFNIKDTYIHWVR	HC(020-038)	2180.0837	1.2	1
76.6	1088.5410	13618352	1.35	DTYIHWVR	HC(031-038)	1088.5403	0.6	0
76.2	829.4442	24354704	2.42	GLEWVAR	HC(044-050)	829.4446	-0.5	0
71.9	1083.5360	19879112	1.97	IYPTNGYTR	HC(051-059)	1083.5349	1.0	0
72.1	1181.6059	3708836	0.37	GRFTISADTSK	HC(066-076)	1181.6041	1.6	1
73.1	968.4819	26423682	2.62	FTISADTSK	HC(068-076)	968.4815	0.4	0
79.3	2260.1184	317550	0.03	FTISADTSKNTAYLQMNSLR	HC(068-087)	2260.1158	1.1	1
81.3	3518.6474	320893	0.03	FTISADTSKNTAYLQMNSLRAEDTAVYYCSR	HC(068-098)	3518.6446	0.8	2
76.8	1309.6451	24858112	2.47	NTAYLQMNSLR	HC(077-087)	1309.6449	0.1	0
79.9	2568.1769	5903991	0.59	NTAYLQMNSLRAEDTAVYYCSR	HC(077-098)	2568.1737	1.2	1
71.6	1276.5392	2994056	0.30	AEDTAVYYCSR	HC(088-098)	1276.5394	-0.1	0
85.3	2783.2545	16863744	1.67	WGGDGFYAMDYWGQGTLVTVSSASTK	HC(099-124)	2783.2537	0.3	0
78.7	1185.6398	68405792	6.79	GPSVFPLAPSSK	HC(125-136)	1185.6394	0.4	0
77.7	1263.6494	36588096	3.63	STSGGTAALGCLVK	HC(137-150)	1263.6493	0.1	0
88.3	6655.2898	221285	0.02	DYFPEPVTVSWNSGALTSGVHTFPAVLQSS GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTK	HC(151-213)	6655.2857	0.6	0
79.8	1374.7171	214690	0.02	VDKKVEPKSCDK	HC(214-225)	1374.7177	-0.4	3
84.9	2729.4093	7103687	0.71	THTCPPCPAPELLGGPSVFLFPPKPK	HC(226-251)	2729.4073	0.7	0
73.4	834.4277	13166738	1.31	DTLMISR	HC(252-258)	834.4269	1.0	0
82.0	2897.4175	220680	0.02	DTLMISRTPEVTCVVVDVSHEDPEVK	HC(252-277)	2897.4151	0.9	1
84.3	4556.2041	547132	0.05	DTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	HC(252-291)	4556.1992	1.1	2
79.3	2081.0013	17858876	1.77	TPEVTCVVVDVSHEDPEVK	HC(259-277)	2080.9987	1.2	0
83.0	3739.7881	18901388	1.88	TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	HC(259-291)	3739.7828	1.4	1
78.6	1676.7966	5991668	0.59	FNWYVDGVEVHNAK	HC(278-291)	1676.7947	1.1	0
84.6	1807.0008	38113724	3.78	VVSVLTVLHQDWLNGK	HC(305-320)	1806.9992	0.9	0
83.3	2227.2022	63210428	6.27	VVSVLTVLHQDWLNGKEYK	HC(305-323)	2227.2001	0.9	1
82.4	2458.3080	2959244	0.29	VVSVLTVLHQDWLNGKEYKCK	HC(305-325)	2458.3043	1.5	2
81.6	2886.5495	183447	0.02	VVSVLTVLHQDWLNGKEYKCKVSNK	HC(305-329)	2886.5426	2.4	3
73.9	837.4964	38694668	3.84	ALPAPIEK	HC(330-337)	837.4960	0.5	0
75.4	1285.6662	875645	0.09	EPQVYTLPPSR	HC(348-358)	1285.6667	-0.4	0
75.6	1903.9366	36378636	3.61	EPQVYTLPPSREEMTK	HC(348-363)	1903.9350	0.8	1
80.5	2989.5263	1849832	0.18	EPQVYTLPPSREEMTKNQVSLTCLVK	HC(348-373)	2989.5253	0.3	2
78.4	1721.8701	179925	0.02	EEMTKNQVSLTCLVK	HC(359-373)	1721.8692	0.6	1
78.9	1103.6013	45037560	4.47	NQVSLTCLVK	HC(364-373)	1103.6009	0.4	0
81.6	2543.1245	29221288	2.90	GFYPSDIAVEWESNGQPENNYK	HC(374-395)	2543.1241	0.2	0
85.7	4398.0307	5032842	0.50	GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK	HC(374-412)	4398.0281	0.6	1
85.9	4954.3531	225838	0.02	GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDK	HC(374-417)	4954.3502	0.6	2
82.8	1872.9144	57538216	5.71	TTPPVLDSDGSFFLYSK	HC(396-412)	1872.9146	-0.1	0
82.6	2429.2370	970108	0.10	TTPPVLDSDGSFFLYSKLTVDK	HC(396-417)	2429.2366	0.2	1
77.8	2986.3744	1042270	0.10	SRWQQGNVFSCSVMHEALHNHYTQK	HC(418-442)	2986.3715	1.0	1
78.5	2743.2427	46302832	4.60	WQQGNVFSCSVMHEALHNHYTQK	HC(420-442)	2743.2384	1.6	0
74.6	659.3487	26985572	2.68	SLSLSPG	HC(443-449)	659.3490	-0.5	0
76.7	1877.8787	2655889	0.26	DIQMTQSPSSLSASVGDR	LC(001-018)	1877.8789	-0.1	0
79.5	2551.2398	9011163	0.89	DIQMTQSPSSLSASVGDRVTITCR	LC(001-024)	2551.2371	1.1	1

RT	Mass	Vol	Vol %	Sequence	Seq Loc.	Tgt. Seq. Mass	Diff. (ppm)	Missed Cleavage
75.6	1989.9932	10501849	1.04	ASQDVNTAVAWYQQKPGK	LC(025-042)	1989.9908	1.2	0
74.8	2286.1771	370620	0.04	ASQDVNTAVAWYQQKPGKAPK	LC(025-045)	2286.1757	0.6	1
84.3	1771.9519	27274394	2.71	LLIYSASFLYSGVPSR	LC(046-061)	1771.9509	0.6	0
85.8	4129.8936	10160642	1.01	SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTK	LC(067-103)	4129.8892	1.1	0
85.3	4599.1803	2980494	0.30	SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIK	LC(067-107)	4599.1792	0.2	1
84.5	4755.2888	6157193	0.61	SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKR	LC(067-108)	4755.2803	1.8	2
82.2	2101.1217	3894068	0.39	RTVAAPSVFIFPPSDEQLK	LC(108-126)	2101.1208	0.4	1
84.0	1945.0220	32666390	3.24	TVAAPSVFIFPPSDEQLK	LC(109-126)	1945.0197	1.2	0
90.2	3666.8789	1457746	0.14	TVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPR	LC(109-142)	3666.8756	0.9	1
85.5	1739.8676	23403588	2.32	SGTASVVCLLNNFYPR	LC(127-142)	1739.8665	0.6	0
75.9	2676.2628	852142	0.08	VQWKVDNALQSGNSQESVTEQDSK	LC(146-169)	2676.2627	0.0	1
80.0	4160.0087	11663837	1.16	VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK	LC(146-183)	4160.0033	1.3	2
79.7	4766.2746	403170	0.04	VQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEK	LC(146-188)	4766.2683	1.3	3
78.8	3618.7073	16391463	1.63	VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK	LC(150-183)	3618.7021	1.5	1
78.6	4224.9705	725845	0.07	VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEK	LC(150-188)	4224.9670	0.8	2
77.3	4490.1265	478190	0.05	VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHK	LC(150-190)	4490.1209	1.3	3
78.9	6290.0188	328711	0.03	VDNALQSGNSQESVTEQDSKDSTYSLSSTLT LSKADYEKHKVYACEVTHQGLSSPVTK	LC(150-207)	6290.0085	1.6	4
79.0	1501.7515	2071466	0.21	DSTYSLSSTLTLSK	LC(170-183)	1501.7512	0.2	0
74.0	2689.3218	547667	0.05	ADYEKHKVYACEVTHQGLSSPVTK	LC(184-207)	2689.3170	1.8	2
73.4	2083.0562	6116953	0.61	HKVYACEVTHQGLSSPVTK	LC(189-207)	2083.0521	2.0	1
75.3	1817.8988	25691042	2.55	VYACEVTHQGLSSPVTK	LC(191-207)	1817.8982	0.3	0

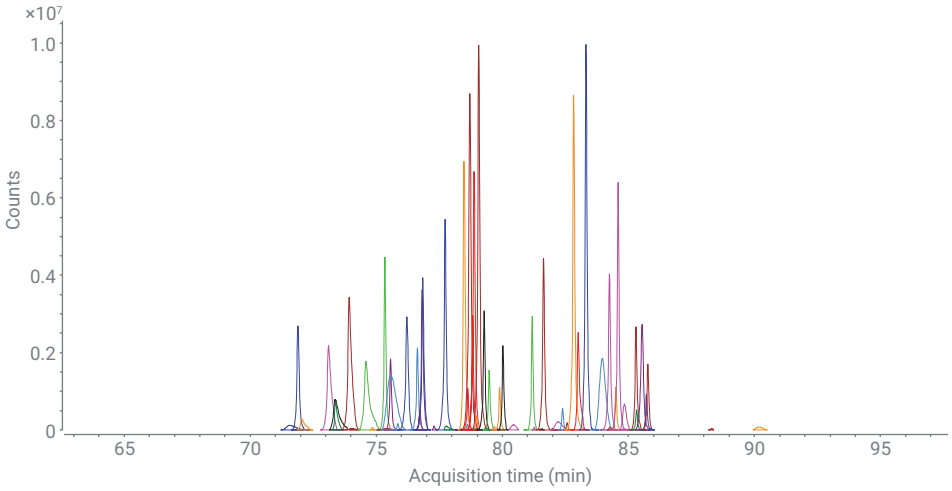

Figure 4. Overlaid RPLC/MS compound chromatograms of MS-identified peptides in the CEX main peak following online peptide mapping.

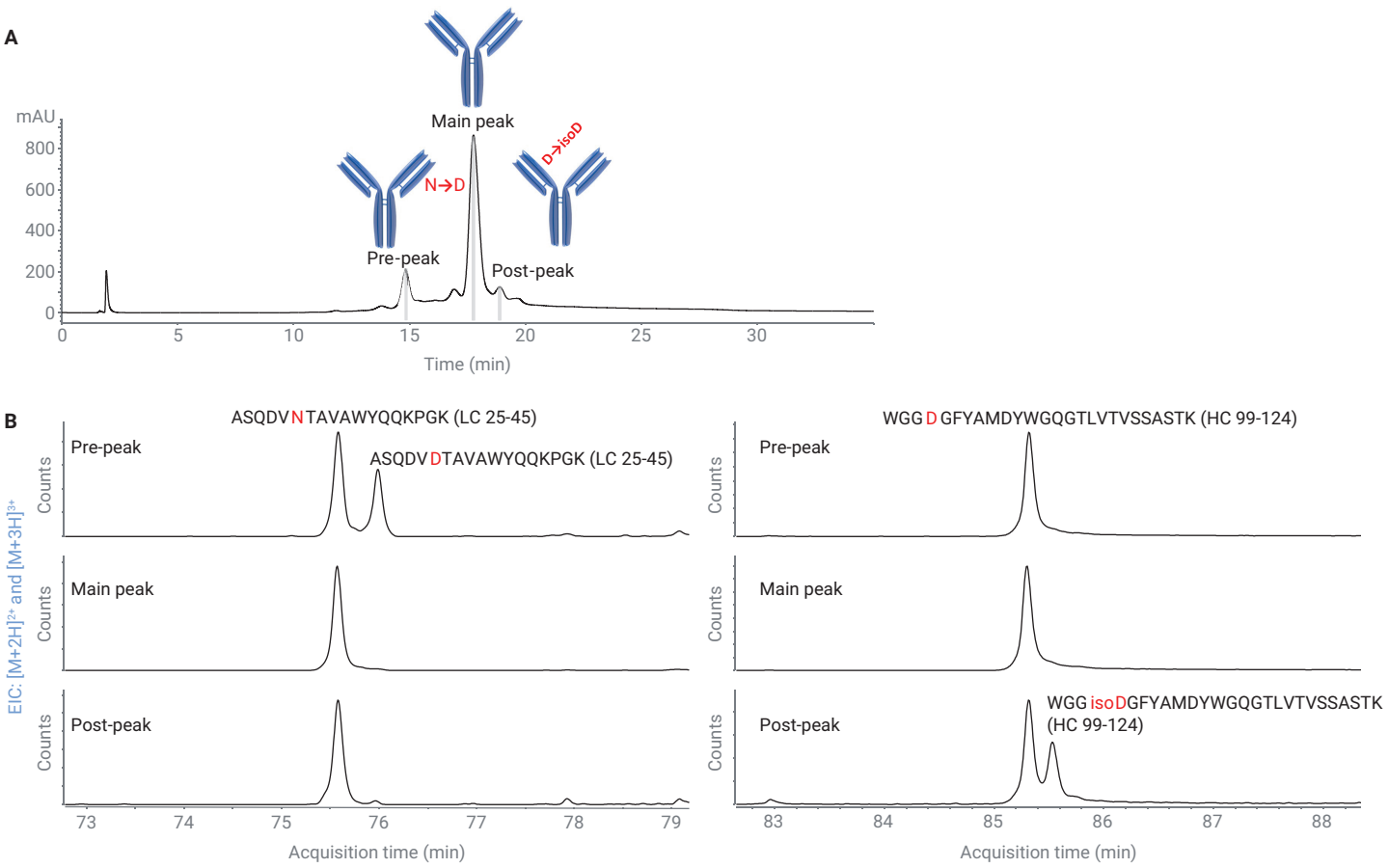

Figure 5. Online peptide mapping of trastuzumab CEX pre-, main-, and post-peaks. (A) the CEX chromatogram and (B) the extracted ion chromatograms of light-chain peptide ASQDVNTAVAWYQQKPGK (LC 25–42), deamidated light-chain peptide ASQDVDTAVAWYQQKPGK (LC 25–42), heavy-chain peptide WGGDGFYAMDYWGQGTLVTVSSASTK (HC 99–124) and isomerized heavy-chain peptide WGGisoDGFYAMDYWGQGTLVTVSSASTK (HC 99–124).

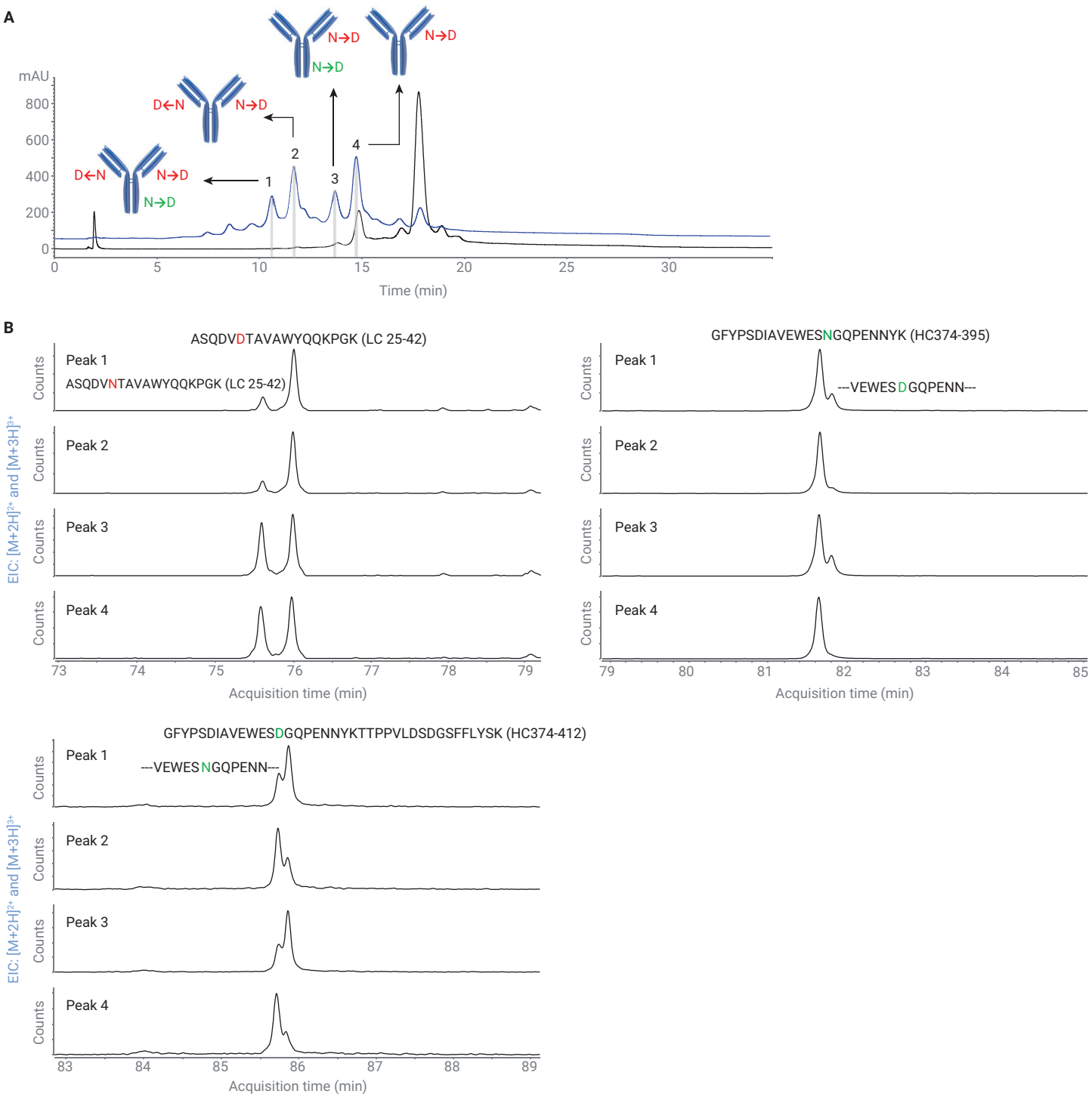

Figure 6. Online peptide mapping of high-pH stressed trastuzumab CEX peaks 1, 2, 3, and 4. (A) the overlaid CEX chromatograms of nonstressed and high-pH stressed trastuzumab and (B) the extracted ion chromatograms of light-chain peptide ASQDVNTAVAWYQQKPGK (LC 25–42), deamidated light-chain peptide ASQDVDTAVAWYQQKPGK (LC 25–42), heavy-chain peptide GFYPSDIAVEWESNGQPENNYK (HC 374–395), deamidated heavy-chain peptide GFYPSDIAVEWESDGQPENNYK (HC 374–395), heavy-chain peptide GFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSK (HC374–412), and deamidated heavy-chain peptide GFYPSDIAVEWESDGQPENNYKTTPPVLDSDGSFFLYSK (HC 374–412).

Conclusion

A fully automated 4D-LC/MS protein analyzer incorporating ^{1}D CEX separation and charge-variant peak collection using multiple heart-cutting, ^{2}D RPLC-based desalting, denaturation, reduction, ^{3}D trypsin digestion, and ^{4}D RPLC/MS-based peptide mapping was described and successfully applied to characterize acidic and basic variants observed in the CEX profile of nonstressed and high-pH stressed trastuzumab. This multidimensional system is based on the InfinityLab 2D-LC Solution and the 6545 LC/Q-TOF system. A variant of this 4D-LC/MS design can readily be configured by replacing CEX in the first dimension by Protein A affinity chromatography, size exclusion chromatography, hydrophobic interaction chromatography, etc.

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