Application Note

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Automated Switching between Peptide and Glycan Mapping with the Agilent 1290 Infinity II LC System

Increased productivity and flexibility in the characterization of monoclonal antibodies

Authors

Gerd Vanhoenacker, Aurélie Terrier, Gianni Vandenborre, Pat Sandra, and Koen Sandra
RIC Biologics
President Kennedypark 26
B-8500 Kortrijk
Belgium

Sonja Schipperges, Sonja Schneider, and Udo Huber
Agilent Technologies
Hewlett-Packard Strasse 8
D-76337 Waldbronn
Germany

Abstract

Glycan and peptide mapping are key analytical methodologies for the structural characterization of therapeutic proteins such as monoclonal antibodies (mAbs). This application note describes glycan and peptide mapping of mAbs performed on the same system without manual intervention upon switching between the modes. The analyses are carried out on an Agilent 1290 Infinity II LC System equipped with a column switching valve, fluorescence detector (FLD), diode array detector (DAD), and quadrupole time-of-flight mass spectrometer (Q-TOF MS). Agilent AdvanceBio Peptide Plus and Glycan Mapping columns are used, the latter in combination with the InstantPC kit, which enables rapid glycan release and labeling. The instrumental setup offers great flexibility and productivity for performing glycan and peptide mapping in one single analytical sequence, as illustrated with various mAb samples.

Introduction

MAbs have emerged as important therapeutics for treatment of life-threatening diseases including cancer and autoimmune and infectious diseases. Together with a huge therapeutic potential comes an immense structural complexity that is highly demanding of analytics. Glycan and peptide mapping are key analytical tools to unravel the structural complexity associated with mAbs.[1–3]

Hydrophilic interaction liquid chromatography (HILIC) combined with FLD and 2-aminobenzamide (2-AB) labeling is considered the golden standard for the analysis of N-glycans originating from mAbs.[4] This method finds its origins in a 25-year-old paper entitled: "A Rapid High-Resolution High-Performance Liquid Chromatographic Method for Separating Glycan Mixtures and Analyzing Oligosaccharide Profiles".[5] The separation was performed on a column packed with 5 µm silica-based particles carrying amide functional groups. At the time, this was considered a normal-phase HPLC method despite the use of an ammonium formate buffer at pH 4.4. Now this LC mode is termed *HILIC*. More recently, Agilent introduced the AdvanceBio Glycan Mapping column, a purpose-built column based on amide chemistry and available in both 1.8 µm fully porous and 2.7 µm superficially porous formats compatible with, respectively, UHPLC (1,200 bar) and HPLC (600 bar). The terms *rapid* and *high resolution* used in the original paper are thereby substantially redefined. Indeed, the reduced diffusion distances provided by sub 2 µm fully porous and sub 3 µm superficially porous particles result in superb, fast separations.[6]

Glycan labeling is carried out to improve chromatographic behavior and detection by either fluorescence and/or mass spectrometry (MS). While labeling with 2-AB is well adapted in many laboratories, it has recently been challenged by other labels that allow more sensitive MS measurements such as procainamide (PC). Historically, sample preparation has been the bottleneck in the analytical process, requiring several days of labor-intensive sample manipulations such as deglycosylation, labeling by reductive amination, and cleanup. Using the latest generation of glycan reagent (InstantPC) reacting with the glycan glycosylamine form and simplified deglycosylation protocol (AdvanceBio Gly-X N-Glycan Prep), processing time can be reduced to less than 1 hour, enabling a significantly higher throughput.[7]

Next to glycan mapping, LC/MS-based peptide mapping is a common and crucial technique performed on mAbs. Important primary structural features such as amino acid sequence, N- and O-glycosylation, glycation, N- and C-terminal processing, deamidation (asparagine, glutamine), aspartate isomerization, and oxidation (methionine, tryptophan) can be readily extracted out of the LC/MS data.[1–3] The complexity associated with tryptic digests of mAbs demands substantial separating power. Reversed-phase liquid chromatography (RPLC) is the method of choice to resolve the vast number of peptides, which have diverse properties in terms of hydrophobicity, size, and charge and exist across a wide dynamic concentration range. The mobile phase additive trifluoroacetic acid (TFA) has historically proven its value to improve peak shape and chromatographic resolution of these basic solutes, but suffers from ionization suppression, thereby reducing MS sensitivity. Replacing the latter with formic acid greatly improves MS sensitivity but diminishes chromatographic performance on traditional reversed-phase columns. The recently introduced Agilent AdvanceBio Peptide Plus column, based on sub-3 µm superficially porous particles featuring a hybrid endcapped C18 stationary phase with a charged surface, provides superior chromatographic performance using the MS-friendly formic acid as mobile phase additive.[8]

Since common biopharma characterization workflows require both glycan and peptide mapping to be carried out, there is interest in realizing this using the same analytical setup within the same analytical sequence. The present application note describes the use of an Agilent 1290 Infinity II LC System equipped with a column switching valve, FLD, DAD, and Q-TOF MS to allow sequential peptide and glycan mapping of mAbs on the AdvanceBio Glycan Mapping and Peptide Plus columns without manual intervention.

Experimental

Instrumentation

An Agilent 1290 Infinity II LC System was used with an Agilent 6545 LC/Q-TOF with an Agilent Jet Stream ESI source. The LC system was composed of the following modules and options and the instrumental configuration and analytical flow path are shown in Figure 1.

- Agilent 1290 Infinity II High-Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B)
  - Sample Thermostat (Option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (MCT) (G7116B)
  - Valve drive (Option #058)
  - Agilent InfinityLab Quick Change Valve, 2-position/6-port, 1,300 bars (G4231C)

- Agilent 1290 Infinity II Diode Array Detector (G7117B)
  - Max-Light Cartridge 10 mm, 1 µL (G4212-6008)
- Agilent 1260 Infinity II Fluorescence Detector (G1321A)
  - Standard flow cell, 8 µL (G1321-60005)

The column switching valve installed in the MCT is used to select the Glycan Mapping or Peptide Plus column and the respective analysis. The peptide-mapping eluate runs through the DAD and MS in series without splitting. The glycan-mapping eluate is split by a ZDV T-piece (Agilent) to FLD and MS. The split is necessary to protect the more fragile FLD flow cell.

Columns

- Agilent AdvanceBio Glycan Map, 2.1 × 150 mm, 2.7 µm (part number 683775-913)
- Agilent AdvanceBio Peptide Plus, 2.1 × 150 mm, 2.7 µm (part number 695775-949)

Software

- Agilent MassHunter workstation (B09.00)
- Agilent MassHunter qualitative analysis (B07.00)
- Agilent MassHunter BioConfirm (B07.00)

Chemicals and reagents

Acetonitrile (HPLC-S), water (ULC/MS), and formic acid (ULC/MS) were obtained from Biosolve. Ammonium formate (LC/MS grade), guanidine-HCl, DL-dithiothreitol (DTT), and 2-iodoacetamide (IAA) were purchased from Sigma. Tris-HCl (1 M, pH 7.5) was obtained from Invitrogen and Trypsin (sequencing grade) from Promega. Type I water was produced from tap water by an arium pro Ultrapure Lab Water System from Sartorius. The AdvanceBio Gly-X with InstantPC Dye kit (GX96-IPC) composed of the Gly-X Deglycosylation Module, Gly-X InstantPC Labeling Module, and Gly-X InstantPC Cleanup Module was obtained from Agilent Technologies.

Samples and sample preparation

A total of 9 mAb samples (originators and biosimilars) were analyzed with the setup. These samples were prepared for the respective analysis as briefly described below. Prepared samples were stored at –20 °C until analyzed. The Agilent AdvanceBio InstantPC CHO mAb N-glycan library (GKPC-020) was used as control sample for the glycan mapping.

Glycan mapping

Samples were prepared according to the protocol described in the user manual. In brief, 40 µg of mAb was denatured at 90 °C for 3 minutes and deglycosylated for 5 minutes at 50 °C using PNGase F. Released glycans were labeled with InstantPC for 1 minute at 50 °C. Labeled N-glycans were subsequently purified using a HILIC solid-phase extraction (SPE) cleanup module.

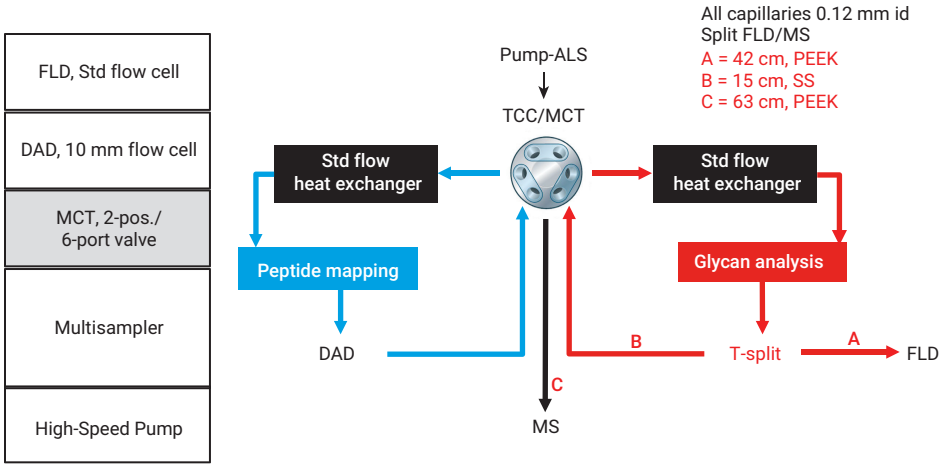

Figure 1. Analytical flow path for automated switching between peptide and glycan analyses.

Method parameters

Glycan Analysis		
LC		
MCT Valve	Position 1 (to glycan column and split FLD-MS)	
Column	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 2.7 µm	
Mobile Phase	A) 50 mM ammonium formate, pH 4.4 in water B) acetonitrile	
Flow Rate	0.5 mL/min	
Gradient	Glycan mapping	Glycan conditioning after switch
	0 to 2 min 80 to 75% B 2 to 38 min 75 to 65% B 38 to 39 min 65 to 40% B 39 to 41.5 min 40 to 80% B 41.5 to 55 min 80% B	0 to 10 min 50% B 10 to 12 min 50 to 80% B 12 to 25 min 80% B
Temperature	40 °C	
Injection	1 µL, 5 °C, Needle wash: 5 s, water/acetonitrile (50/50) in flush port	
Detection		
FLD		
Wavelength	Ex 285 nm, Em 345 nm	
Peak Width	>0.05 min (1 s response time, 9.26 Hz)	
PMT Gain	10	
MS		
Diverter Valve to MS	5 to 39 min	
Source		
Drying Gas Temperature	150 °C	
Drying Gas Flow	8 L/min	
Sheath Gas Temperature	300 °C	
Sheath Gas Flow	10 L/min	
Nebulizer Pressure	30 psi	
Capillary Voltage	2,500 V	
Nozzle Voltage	500 V	
Fragmentor	120 V	
Acquisition		
Ion Polarity	Positive	
Acquisition Mode	Extended dynamic range (2 GHz)	
Mass Range	*m/z* 600 to 3,000	
Scan Rate	1 spectrum/s	
Reference Mass	Disabled	

Peptide Mapping		
LC		
MCT Valve	Position 2 (to peptide column and DAD/MS)	
Column	Agilent AdvanceBio Peptide Plus, 2.1 × 150 mm, 2.7 µm	
Mobile Phase	A) 0.1% formic acid in water B) 0.1% formic acid in acetonitrile	
Flow Rate	0.3 mL/min	
Gradient	Peptide mapping	Peptide conditioning after switch
	0 to 1 min 2% B 1 to 41 min 2 to 47% B 41 to 42 min 47 to 95% B 42 to 47 min 95% B 47 to 48 min 95 to 2% B 48 to 55 min 2% B	0 to 15 min 50% B 15 to 16 min 50 to 95% B 16 to 20 min 95% B 20 to 21 min 95 to 2% B 21 to 30 min 2% B
Temperature	50 °C	
Injection	5 µL, 5 °C, Needle wash: 5 s, water/acetonitrile (50/50) in flush port	
Detection		
DAD		
Wavelength	214/8 nm, 220/8 nm, 280/8 nm	
Peak Width	>0.025 min (0.5 s response time, 10 Hz)	
MS		
Diverter Valve to MS	0 to 42 min	
Source		
Drying Gas Temperature	325 °C	
Drying Gas Flow	12 L/min	
Sheath Gas Temperature	300 °C	
Sheath Gas Flow	12 L/min	
Nebulizer Pressure	45 psi	
Capillary Voltage	4,000 V	
Nozzle Voltage	0 V	
Fragmentor	175 V	
Acquisition		
Ion Polarity	Positive	
Acquisition Mode	Extended dynamic range (2 GHz)	
Reference Mass	Disabled	
Acquisition MS		
Mass Range	*m/z* 100 to 3,000	
Scan Rate	2 spectra/s	
Acquisition MS/MS		
Auto MS/MS Mass Range	*m/z* 100 to 3,000	
Auto MS/MS Scan Rate	4 spectra/s	
Isolation Width	Medium (~*m/z* 4)	
Charge State Preference	2/3/>3/Unk	
Collision Energy	All: 4 (slope) and −4.8 (offset)	
Max Precursors/Cycle	5	
Prec. Threshold Abs/Rel%	1,000/0.010	
Isotope Model	Peptides	
Active Exclusion	After two spectra for 0.5 min	
Sort Precursors	Abundance only	
Purity	Stringency: 100%, cutoff: 30%	

Peptide mapping

In brief, 100 µg of mAb was denatured using 3 M guanidine-HCl in 100 mM Tris-HCl pH 7.5, reduced with 5 mM DTT for 30 minutes at 60 °C, and alkylated for 1 hour at 37 °C using IAA. The samples were subsequently desalted by gel filtration prior to overnight trypsin digestion at 37 °C using an enzyme:protein ratio of 1:25.

Analytical sequence with method switching

The analyses described below were carried out in one single analytical instance without operator intervention. Automated switching from peptide to glycan mapping and back was carried out multiple times within this experiment. Upon switching from one method to another, an intermediate method was run to condition the system and column for the subsequent analysis.

Line	Sample	Method
1	Blank	Glycan
2	Glycan CHO control	Glycan
3	Glycan CHO control	Glycan
4	Glycan CHO control	Glycan
5	Trastuzumab originator	Glycan
6	Trastuzumab biosimilar (control)	Glycan
7	Trastuzumab biosimilar (4x)	Glycan
8	Trastuzumab biosimilar (8x)	Glycan
9	Trastuzumab biosimilar (16x)	Glycan
10	Trastuzumab biosimilar (24x)	Glycan
11	Blank	Transfer Glycan to Peptide
12	Blank	Peptide
13	Trastuzumab originator	Peptide
14	Trastuzumab originator	Peptide
15	Trastuzumab originator	Peptide
16	Trastuzumab biosimilar (control)	Peptide
17	Trastuzumab biosimilar (4x)	Peptide
18	Trastuzumab biosimilar (8x)	Peptide
19	Trastuzumab biosimilar (16x)	Peptide
20	Trastuzumab biosimilar (24x)	Peptide
21	Blank	Transfer Peptide to Glycan
22	Blank	Glycan
23	Rituximab originator	Glycan
24	Rituximab biosimilar 1	Glycan
25	Rituximab biosimilar 2	Glycan
26	Blank	Transfer Glycan to Peptide
27	Blank	Peptide
28	Rituximab originator	Peptide
29	Rituximab biosimilar 1	Peptide
30	Rituximab biosimilar 2	Peptide
	Storage	

Results and discussion

Glycan mapping

The analysis sequence started with a set of glycan mapping experiments. Figure 2 shows the simultaneously acquired FLD and MS traces of the triplicate HILIC analysis of the InstantPC CHO mAb N-glycan library obtained on the superficially porous 2.7 µM AdvanceBio Glycan Mapping column. High resolution and, hence, great detail in N-glycosylation is obtained both in the FLD and MS profile. InstantPC-labeled glycans elute based on their polymerization degree (i.e., the higher the number of glycosidic bonds, the greater the retention). Sufficient selectivity differences exist to separate compounds with the same polymerization degree including isomeric compounds such as G1F[3] and G1F[6], which differ in the positioning of the galactose residue either on the α1-3 or α1-6 branch of the complex type glycans. Figure 3 shows representative MS spectra of selected minor and major InstantPC-labeled N-glycans. Clean and intense spectra are observed, which can be explained by the high proton affinity of the PC basic tail (tertiary amine). Tables 1 and 2 present, respectively, the MS and FLD retention time, and also peak area and peak area% precision obtained upon analyzing the InstantPC-labeled N-glycans in triplicate. Excellent relative standard deviation (RSD) values are shown. Peak area% slightly differs between MS and FLD detection, which can be explained by MS ionization differences. As such, the MS trace is mainly used for identification purposes and the FLD trace for quantification purposes.

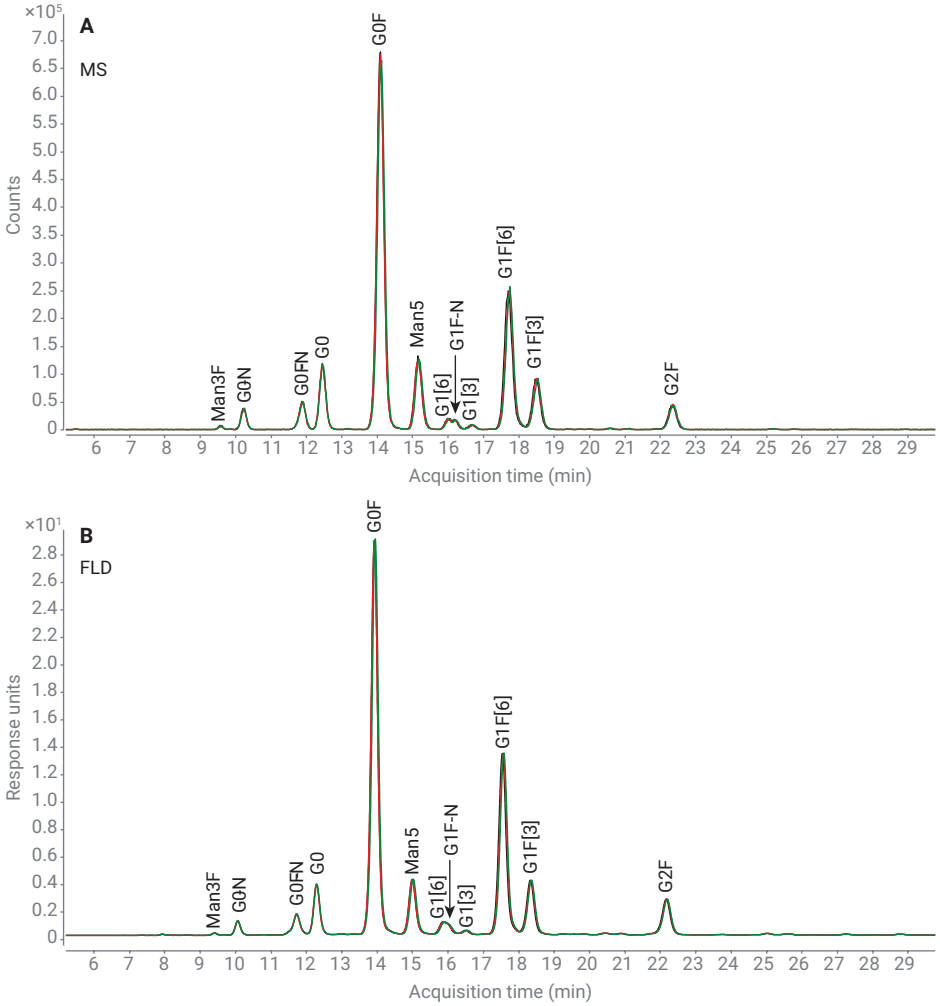

Figure 2. Triplicate HILIC-MS (A) and HILIC-FLD (B) chromatograms of the Agilent AdvanceBio InstantPC CHO mAb N-glycan library on the superficially porous 2.7 µm Agilent AdvanceBio Glycan Mapping column. For the MS trace, the base peak chromatogram (BPC) is presented. Glycan nomenclature according to Agilent publication 5994-2202EN.[9]

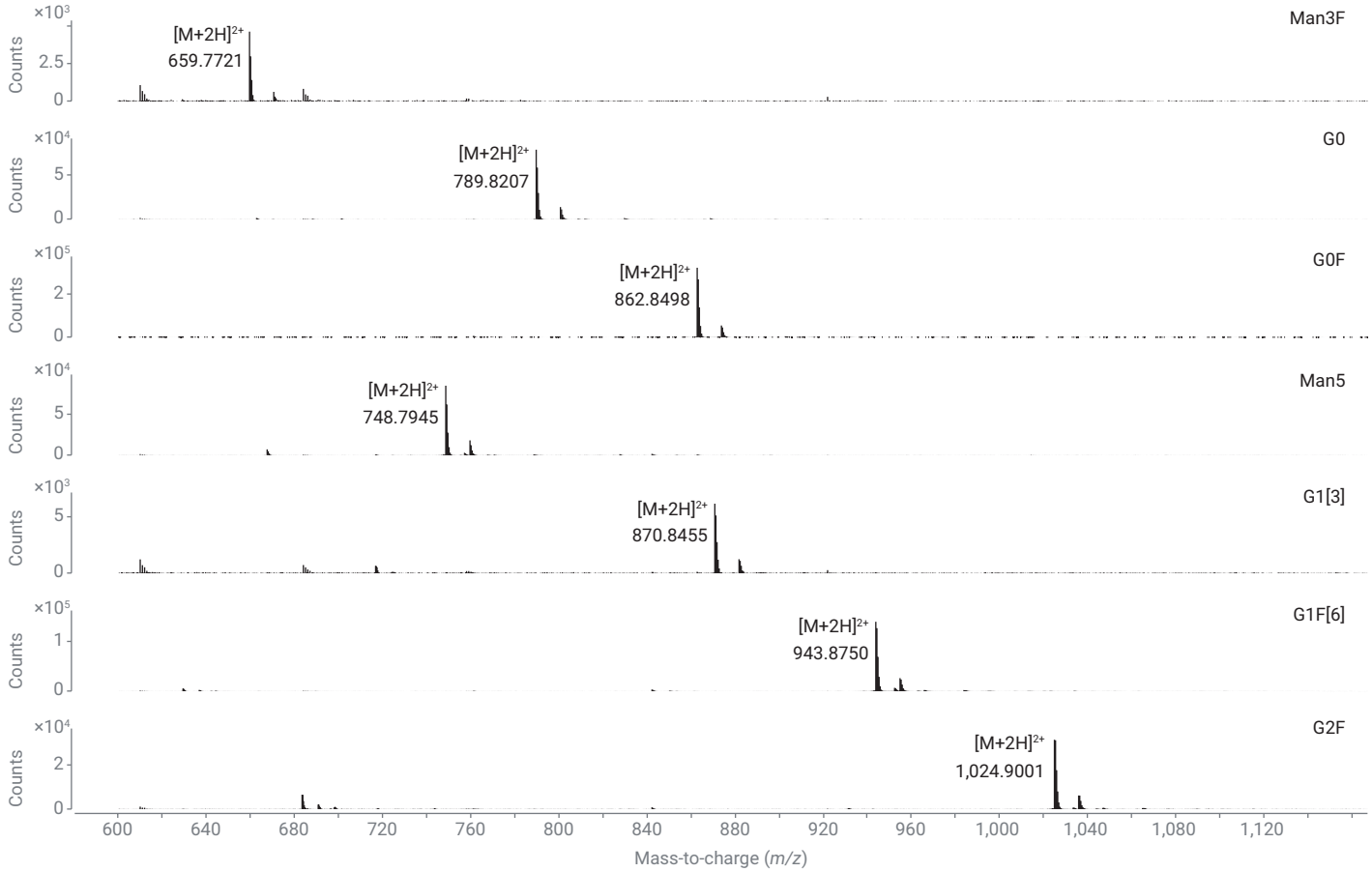

Figure 3. MS spectra corresponding to selected minor and major glycan peaks observed in the Agilent AdvanceBio InstantPC CHO mAb N-glycan library as presented in Figure 2.

Table 1. MS retention time, peak area, and peak area% RSD values for the triplicate analysis of the Agilent AdvanceBio InstantPC CHO mAb N-glycan library.

	Retention Time (min)				Area				Area%			
	Inj1	Inj2	Inj3	RSD%	Inj1	Inj2	Inj3	RSD%	Inj1	Inj2	Inj3	RSD%
Man3F	9.57	9.57	9.61	0.24	63,179	60,303	57,197	4.97	0.29	0.29	0.27	3.77
G0-N	10.22	10.23	10.23	0.06	438,962	407,010	425,035	3.78	2.04	1.93	2.03	3.12
G0F-N	11.89	11.89	11.89	0.00	677,744	650,350	641,947	2.85	3.15	3.08	3.06	1.58
G0	12.45	12.45	12.47	0.09	1,482,185	1,491,906	1,493,663	0.42	6.90	7.07	7.12	1.67
G0F	14.09	14.09	14.11	0.08	10,089,817	9,847,418	9,847,909	1.41	46.96	46.64	46.97	0.40
Man5	15.15	15.15	15.18	0.11	1,879,904	1,847,418	1,829,999	1.37	8.75	8.75	8.73	0.14
G1[6]	15.99	16.01	16.04	0.16	225,860	242,203	235,501	3.50	1.05	1.15	1.12	4.51
G1F-N	16.18	16.19	16.21	0.09	201,264	181,045	186,695	5.50	0.94	0.86	0.89	4.45
G1[3]	16.67	16.69	16.71	0.12	121,670	113,030	110,855	4.97	0.57	0.54	0.53	3.69
G1F[6]	17.70	17.73	17.75	0.14	4,091,308	4,068,838	4,015,420	0.96	19.04	19.27	19.15	0.60
G1F[3]	18.49	18.51	18.53	0.11	1,486,906	1,477,715	1,412,363	2.79	6.92	7.00	6.74	1.96
G2F	22.33	22.35	22.37	0.09	727,378	726,061	710,476	1.30	3.39	3.44	3.39	0.88

Table 2. FLD retention time, peak area, and peak area% RSD values for the triplicate analysis of the Agilent AdvanceBio InstantPC CHO mAb N-glycan library.

	Retention Time (min)				Area				Area%			
	Inj1	Inj2	Inj3	RSD%	Inj1	Inj2	Inj3	RSD%	Inj1	Inj2	Inj3	RSD%
Man3F	9.39	9.40	9.41	0.11	1.67	1.71	1.71	1.36	0.20	0.20	0.20	1.55
G0-N	10.05	10.06	10.06	0.06	11.23	11.31	11.37	0.62	1.33	1.34	1.35	0.80
G0F-N	11.72	11.72	11.73	0.05	22.13	22.16	22.29	0.38	2.62	2.63	2.64	0.53
G0	12.28	12.28	12.29	0.05	46.05	45.94	45.91	0.16	5.44	5.45	5.45	0.04
G0F	13.92	13.92	13.94	0.08	386.64	386.37	385.08	0.22	45.71	45.82	45.67	0.16
Man5	14.98	14.99	15.02	0.14	54.34	54.46	54.69	0.33	6.42	6.46	6.49	0.49
G1[6] + G1F-N	15.86	15.88	15.90	0.13	17.82	18.10	18.49	1.86	2.11	2.15	2.19	2.02
G1[3]	16.50	16.52	16.53	0.09	4.47	4.41	4.44	0.68	0.53	0.52	0.53	0.53
G1F[6]	17.53	17.56	17.58	0.14	198.71	196.70	196.76	0.58	23.49	23.33	23.34	0.39
G1F[3]	18.33	18.34	18.36	0.08	57.76	57.38	57.61	0.33	6.83	6.80	6.83	0.23
G2F	22.17	22.18	22.19	0.05	45.09	44.74	44.74	0.45	5.33	5.31	5.31	0.26

Figure 4 shows the HILIC-FLD N-glycan profiles of a trastuzumab originator (Herceptin) and a biosimilar in development on the 2.7 µm AdvanceBio Glycan Mapping column. The biosimilar was harvested from the CHO cell culture medium using the Bio-Monolith protein A column as described in earlier application notes.[10,11] The same type of complex N-glycans are observed on both the originator and candidate biosimilar, but quantitative differences are revealed with an overexpression of G0F species on the biosimilar. Since glycosylation is a critical quality attribute, this undergalactosylation does not make the product similar enough to be considered by regulatory authorities as a Herceptin biosimilar.

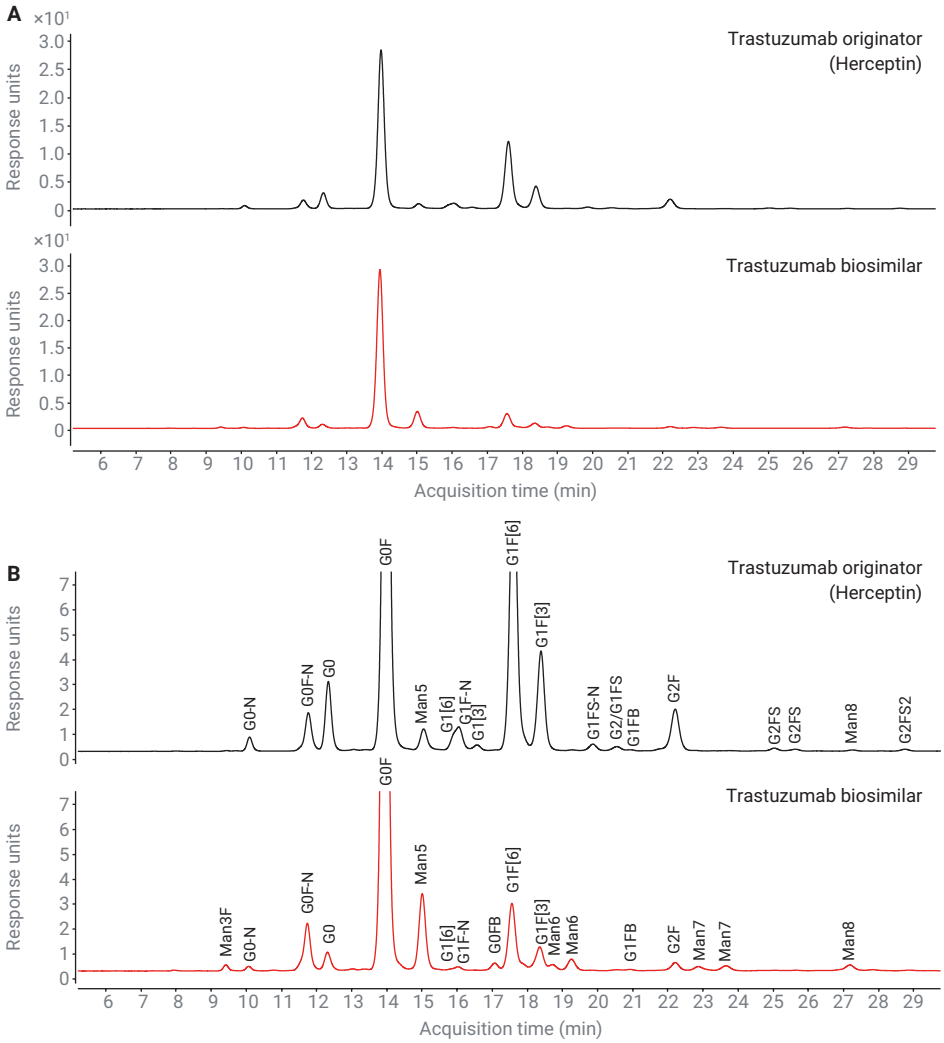

Figure 4. HILIC-FLD chromatograms (unzoomed and zoomed) of the InstantPC-labeled N-glycans enzymatically released from trastuzumab originator and Protein A purified candidate biosimilar. Glycan nomenclature according to Agilent publication 5994-2202EN.[9]

Figure 5 shows the N-glycan profiles obtained by growing the biosimilar producing CHO clone at different galactose, uridine, and manganese chloride concentrations (4x, 8x, 16x, and 24x).[12] These are the substrates and activator of the galactosyltransferase responsible for donating galactose residues to G0F and G1F acceptors. The relative intensity of the N-glycans in the different samples is presented in Figure 6. It is observed that the ratio G1F/G0F increases with increasing concentration of galactose, uridine, and manganese chloride. From these results, it can be concluded that conditions can be found that enable fitting the glycosylation of the biosimilar within the originator specifications.

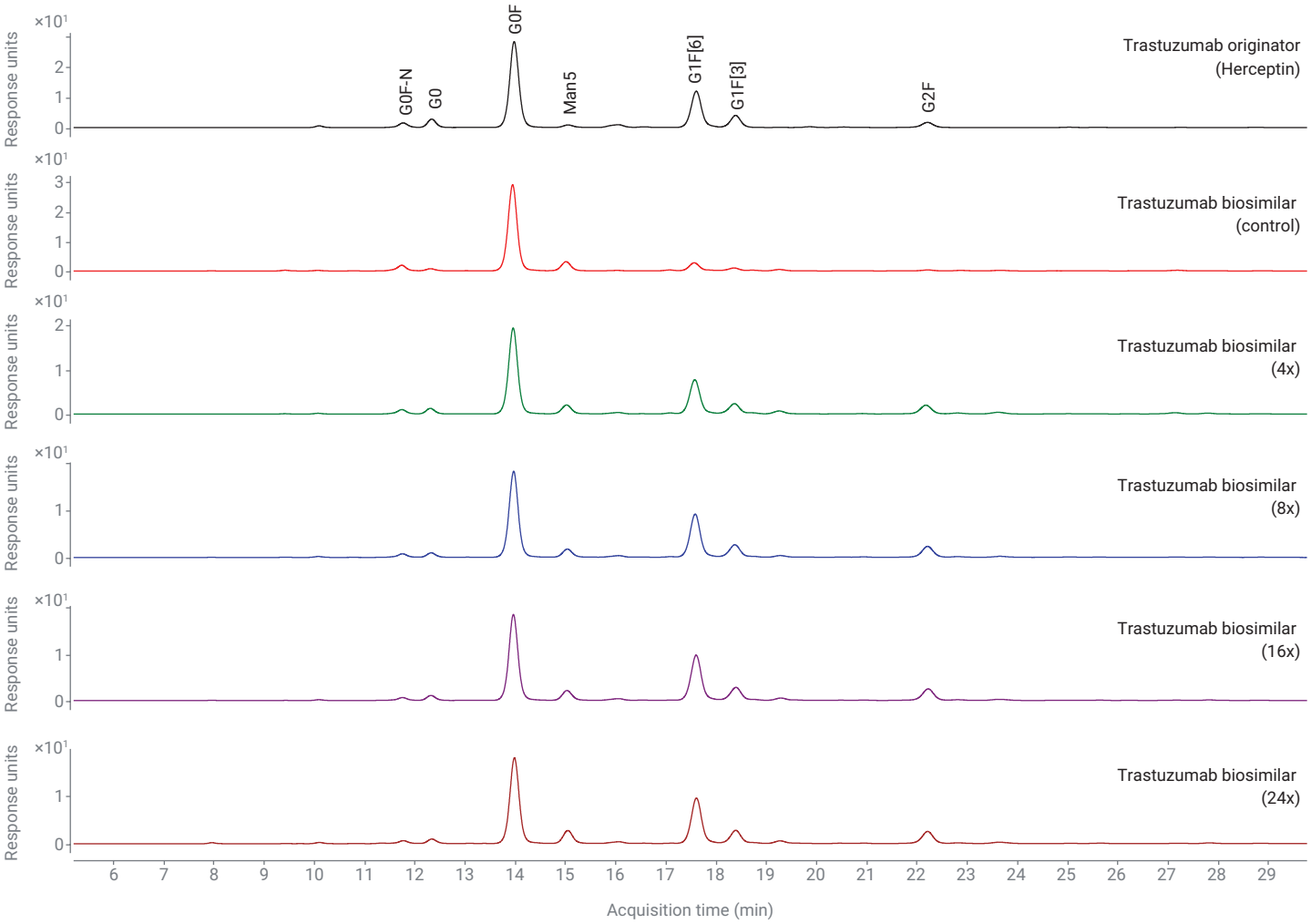

Figure 5. HILIC-FLD N-glycan profiles of the trastuzumab originator and candidate biosimilar obtained by growing the CHO clone at different galactose, uridine, and manganese chloride concentrations.

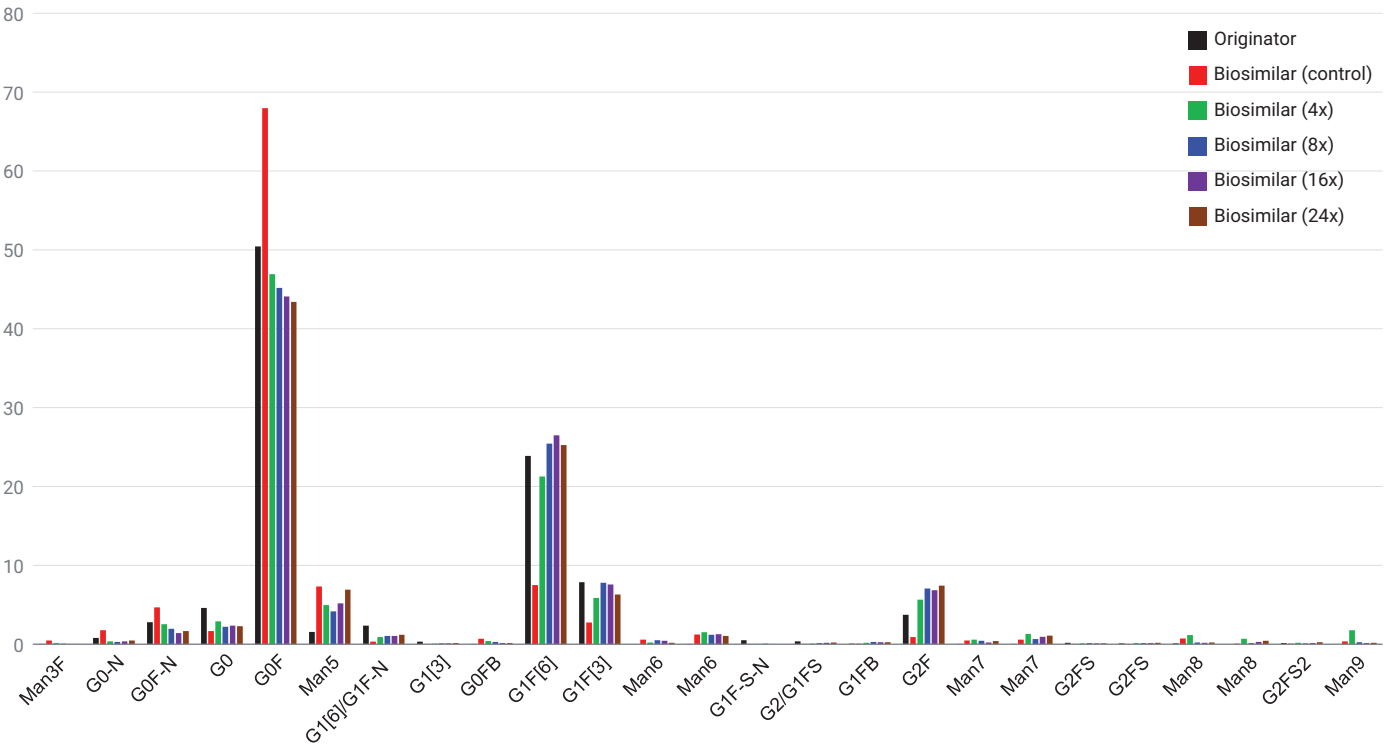

Figure 6. Relative intensity of the glycans in the trastuzumab originator and biosimilars obtained by growing the CHO clone at different galactose, uridine, and manganese chloride concentrations.

Figure 7 shows the HILIC-FLD N-glycan profiles of a rituximab originator (Mabthera) and two approved biosimilars analyzed on the setup following a series of peptide mapping experiments. High-quality data are obtained and the existence of various complex and high mannose type N-glycans is revealed. The observed N-glycans are similar between samples from a qualitative perspective but differ slightly from a quantitative perspective as shown in Figure 8.

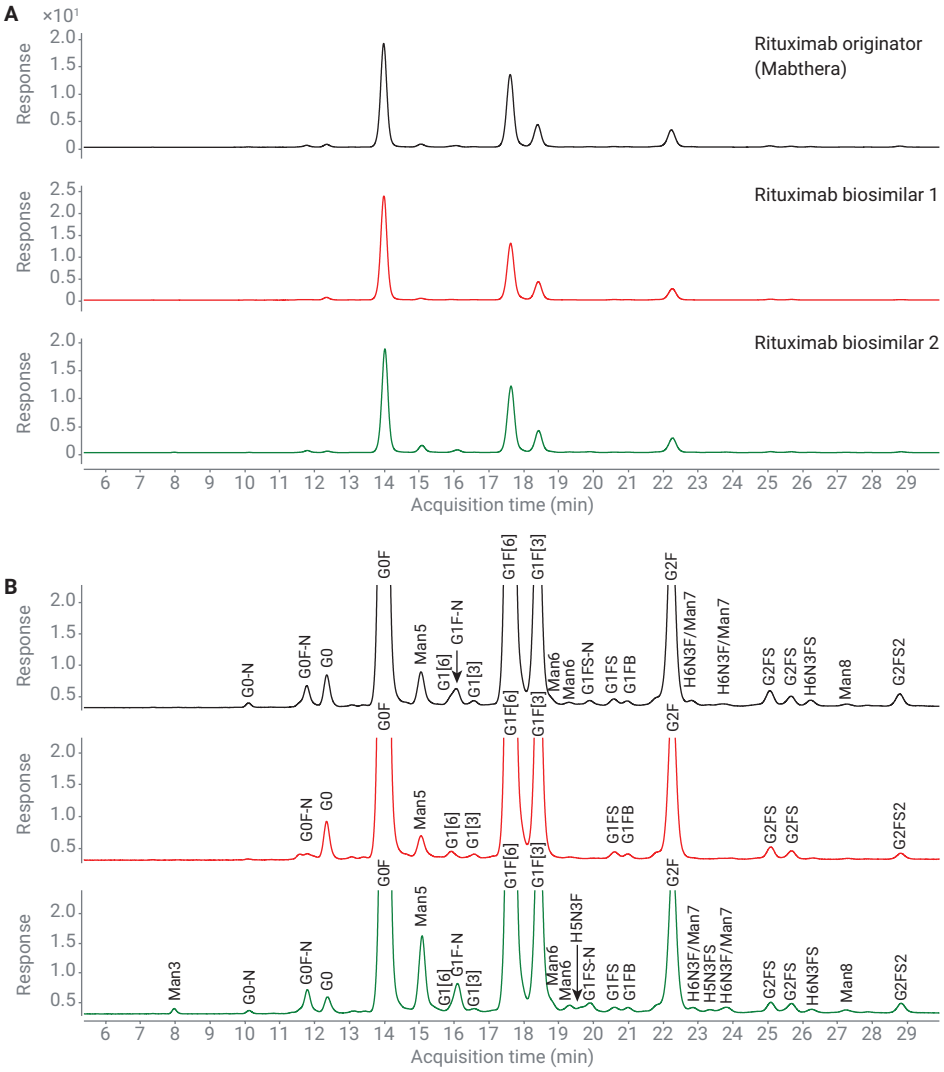

Figure 7. HILIC-FLD chromatograms of the InstantPC-labeled N-glycans enzymatically released from rituximab originator (Mabthera) and approved biosimilars. Glycan nomenclature according to Agilent publication 5994-2202EN.[9] H, N, F, and S correspond to, respectively, hexose, N-acetylglucosamine, fucose, and N-acetylneuraminic acid.

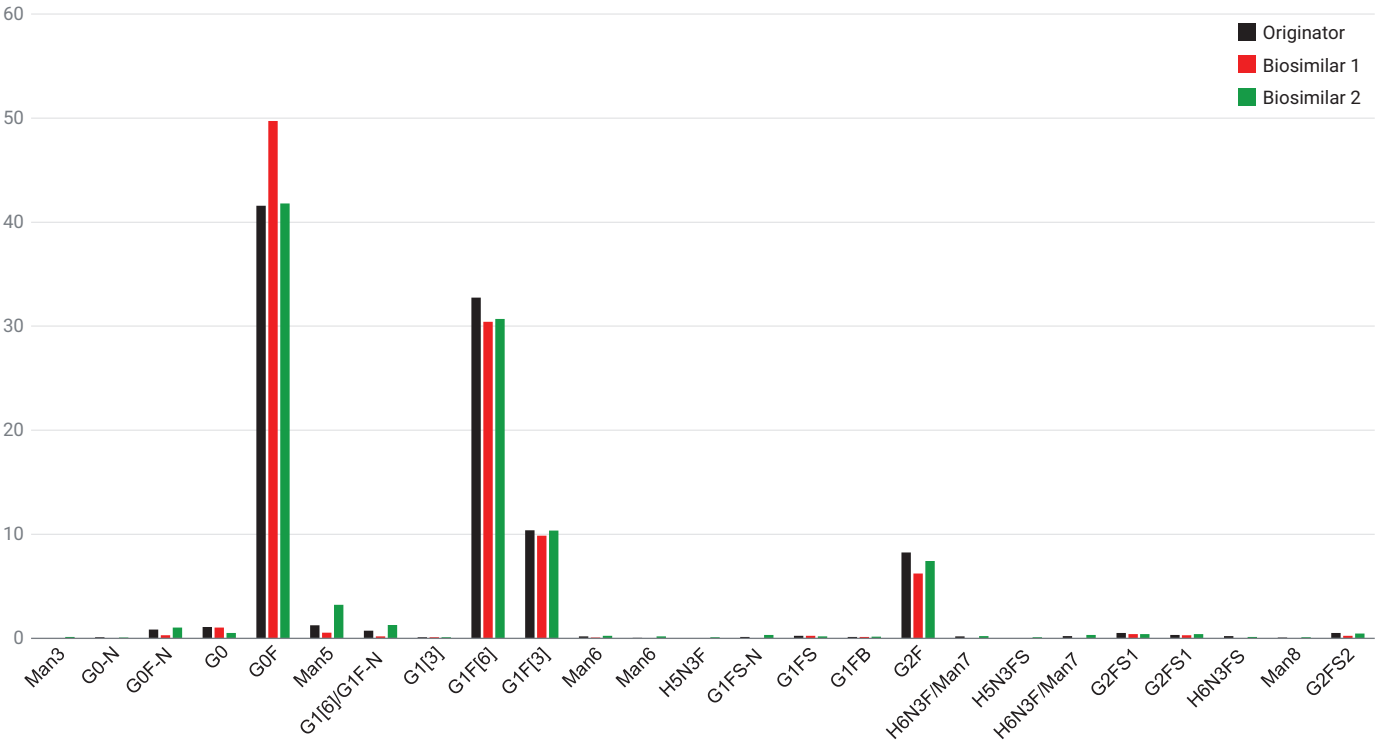

Figure 8. Relative intensity of the glycans in the rituximab originator (Mabthera) and approved biosimilars.

Peptide mapping

Following the glycan mapping experiments of the trastuzumab originator and candidate biosimilar samples, the setup was conditioned for peptide mapping. Figure 9 shows the replicate LC/MS analysis of the trastuzumab originator tryptic digests on the superficially porous 2.7 µm AdvanceBio Peptide Plus column. Over 98.5% sequence coverage is consistently obtained and post-translational modifications such as glycosylation, oxidation, deamidation, N-terminal cyclization, and C-terminal lysine truncation, amongst others, precisely determined. Table 3 presents precision data on selected modified and nonmodified peptides.

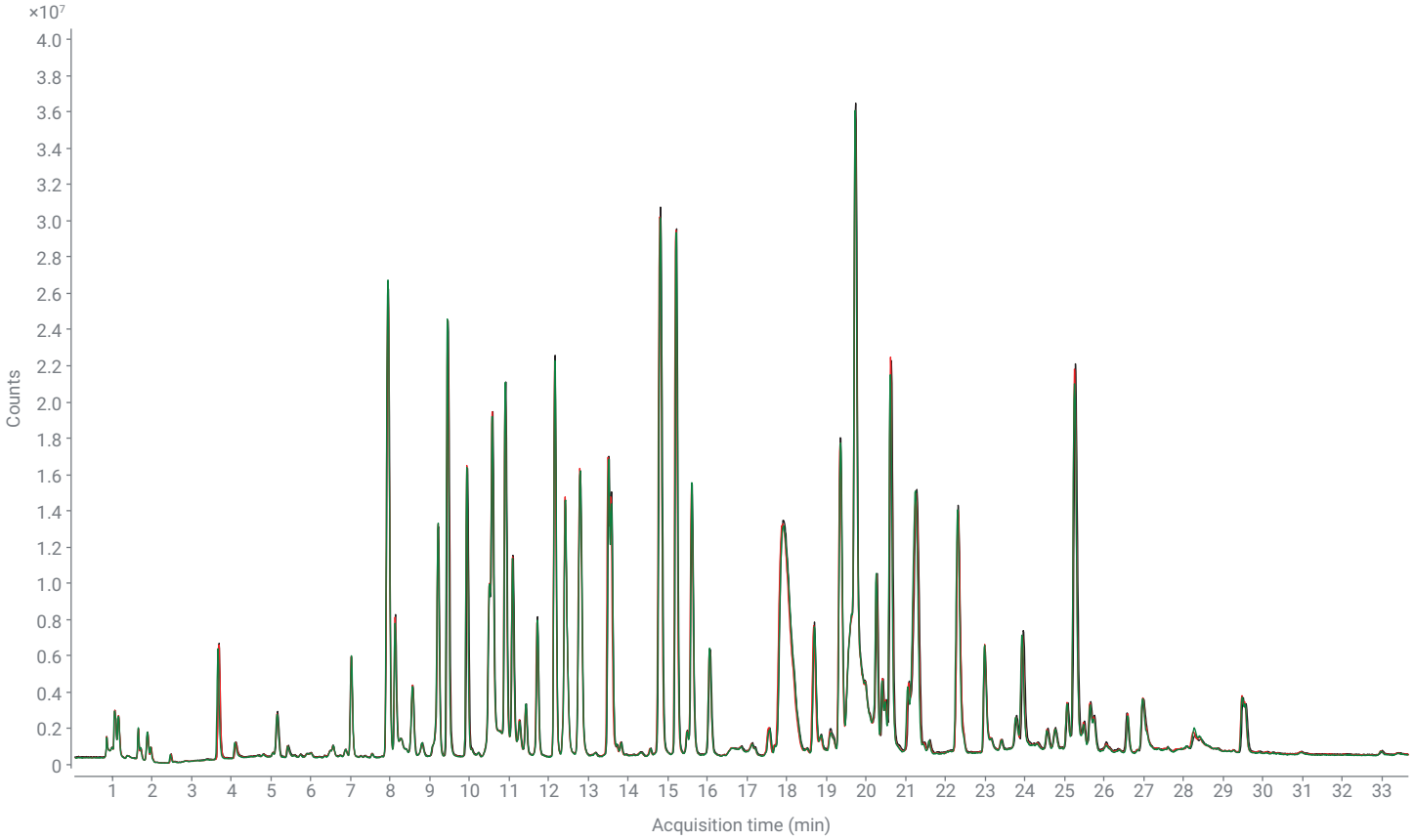

Figure 9. LC/MS TIC traces of trastuzumab originator tryptic digest analyzed in triplicate on the superficially porous 2.7 µm Agilent AdvanceBio Peptide Plus column.

Table 3. LC/MS retention time and peak area precision data (n = 3) of selected trastuzumab originator peptides.

Peptide	Modification	Location	Retention Time (min)				Area			
			Inj1	Inj2	Inj3	RSD%	Inj1	Inj2	Inj3	RSD%
EVQLVESGGGLVQPGGSLR		Hc(001-019)	15.22	15.20	15.21	0.07	21,543,578	21,568,171	21,271,169	0.77
EVQLVESGGGLVQPGGSLR	Pyroglutamate (E1)	Hc(001-019)	19.73	19.73	19.73	0.00	227,186	225,778	230,268	1.01
QAPGK		Hc(039-043)	1.07	1.07	1.07	0.00	1,270,928	1,265,079	1,224,080	2.04
IYPTNGYTR		Hc(051-059)	7.03	7.02	7.02	0.08	5,876,176	5,956,977	5,921,920	0.68
IYPTNGYTR	Deamidation (N55)	Hc(051-059)	7.82	7.82	7.81	0.07	75,634	77,128	78,682	1.98
IYPTNGYTR	Deamidation (N55)	Hc(051-059)	8.28	8.27	8.28	0.07	1,929,925	1,908,310	1,914,802	0.58
IYPTNGYTR	Deamidation (N55)	Hc(051-059)	9.12	9.12	9.12	0.00	312,718	311,721	316,366	0.78
VEPK		Hc(218-221)	1.07	1.07	1.07	0.00	323,901	330,331	316,162	2.19
THTCPPCPAPELLGGPSVFLFPPKPK		Hc(226-251)	17.92	17.89	17.91	0.09	17,363,016	17,015,571	16,981,394	1.23
DTLMISR	Oxidation (N255)	Hc(252-258)	7.55	7.55	7.56	0.08	255,300	259,876	251,521	1.64
DTLMISR		Hc(252-258)	9.22	9.21	9.21	0.06	11,595,004	11,749,527	11,800,546	0.91
TPEVTCVVVDVSHEDPEVK		Hc(259-277)	14.82	14.80	14.81	0.07	37,674,640	37,729,504	37,213,192	0.76
EEQYNSTYR		Hc(296-304)	4.90	4.90	4.88	0.24	16,934	16,324	17,492	3.45
EEQYNSTYR	G0F (N300)	Hc(296-304)	5.17	5.16	5.16	0.11	110,472	105,261	107,652	2.42
VSNK		Hc(326-329)	1.07	1.07	1.07	0.00	356,697	359,808	350,322	1.36
EPQVYTLPPSR		Hc(348-358)	10.55	10.55	10.60	0.27	10,424,387	10,429,544	10,360,028	0.37
NQVSLTCLVK		Hc(364-373)	12.16	12.15	12.16	0.05	33,731,609	33,604,345	33,486,848	0.36
NQVSLTCLVK	Deamidation (N364)	Hc(364-373)	14.37	14.35	14.37	0.08	182,196	185,270	186,655	1.24
NQVSLTCLVK	Deamidation (N364)	Hc(364-373)	15.65	15.64	15.64	0.04	812,465	809,927	811,612	0.16
TTPPVLDSDGSFFLYSK		Hc(396-412)	19.36	19.35	19.36	0.03	17,785,338	17,610,740	17,615,308	0.56
SLSLSPG	K-truncation (K450)	Hc(443-449)	10.51	10.50	10.50	0.05	15,601,783	15,393,916	15,630,806	0.83
SLSLSPGK		Hc(443-450)	6.49	6.49	6.49	0.00	118,037	112,418	112,434	2.83
SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTK		Lc(067-103)	22.33	22.33	22.31	0.05	2,007,854	1,993,328	2,014,568	0.54
VEIK		Lc(104-107)	1.65	1.65	1.65	0.00	1,635,756	1,644,870	1,694,386	1.90
TVAAPSVFIFPPSDEQLK		Lc(109-126)	19.65	19.64	19.64	0.03	10,494,089	10,075,113	10,090,599	2.32
VDNALQSGNSQESVTEQDSK		Lc(150-169)	8.14	8.13	8.13	0.07	3,067,119	2,974,891	2,835,943	3.93
VYACEVTHQGLSSPVTK		Lc(191-207)	9.47	9.45	9.45	0.12	23,190,381	23,107,936	22,919,049	0.60

The LC/MS TICs associated with the tryptic digests of rituximab originator and approved biosimilars are shown in Figure 10. Extracted ion chromatograms (EICs) of selected modified and nonmodified peptides are presented in Figure 11. Differences in light chain and heavy chain N-terminal cyclization, heavy chain C-terminal lysine truncation and proline amidation, as well as N-glycosylation site occupancy are revealed between the samples. For the latter measurement, an interesting selectivity is obtained on the AdvanceBio Peptide Plus column (i.e., the nonglycosylated peptide (EEQYNSTYR) elutes in between the glycopeptides decorated with high-mannose and complex-type N-glycans). These measurements were performed following the glycan mapping of the rituximab samples.

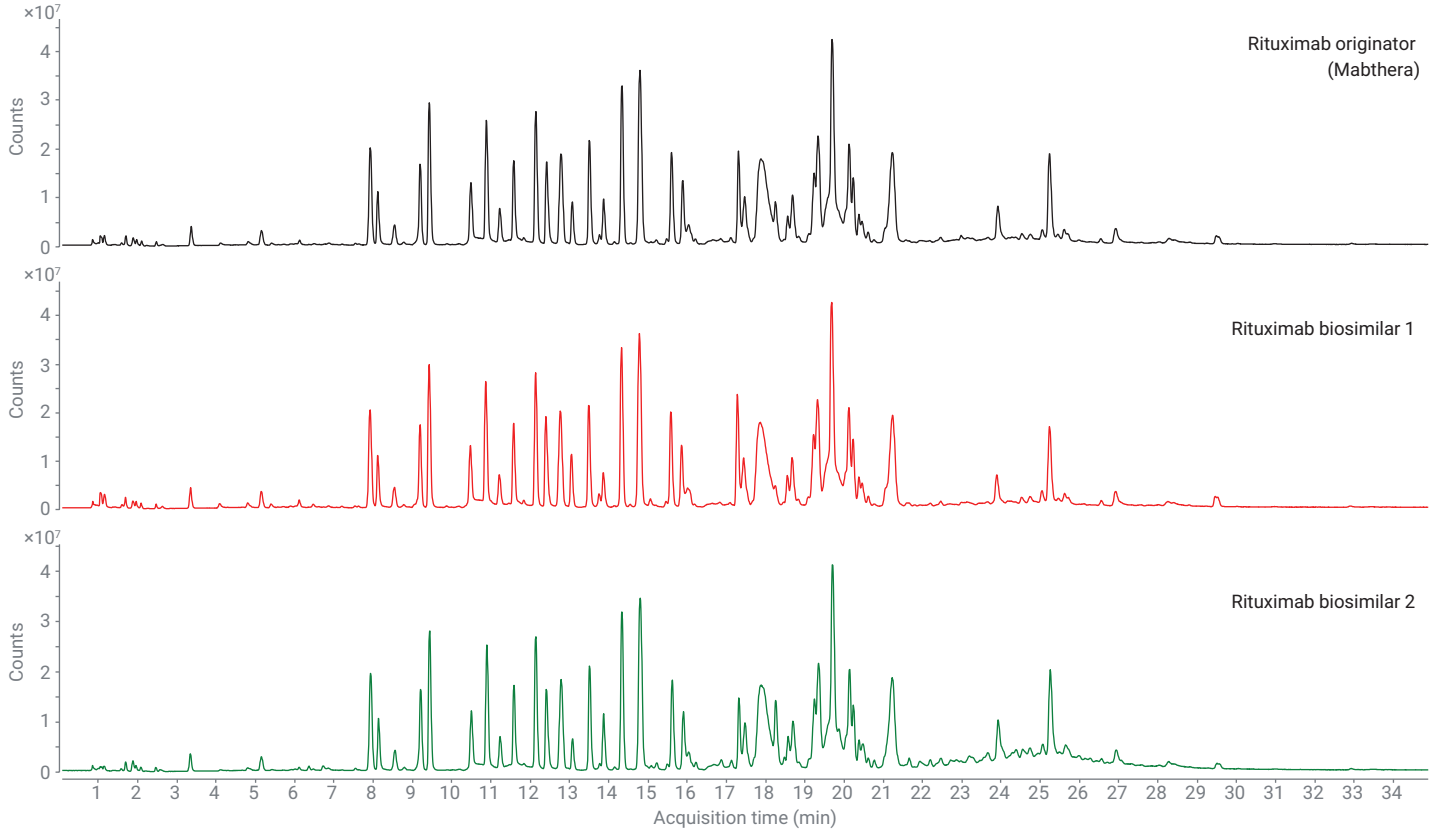

Figure 10. LC/MS peptide maps (TIC) of rituximab originator (Mabthera) and approved biosimilars.

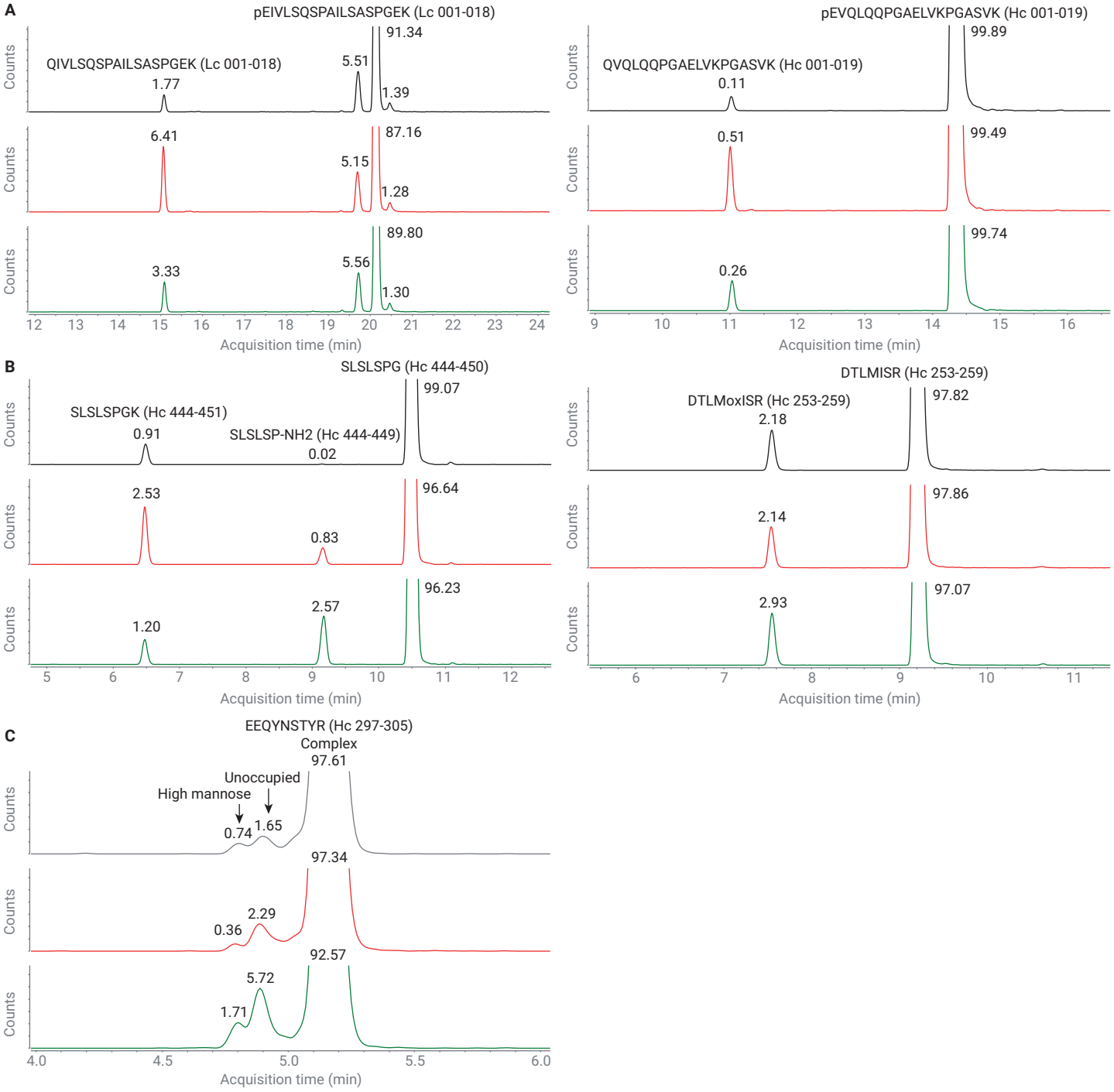

Figure 11. EICs of selected modified and non-modified peptides observed in rituximab originator (Mabthera) (A) and approved biosimilar 1 (B) and biosimilar 2 (C). EICs were extracted at 10 ppm mass accuracy. Peak annotation shows the summed area %.

Conclusion

An Agilent 1290 Infinity II LC System equipped with a column switching valve, fluorescence detector (FLD), diode-array detector (DAD), and quadrupole time-of-flight mass spectrometer (Q-TOF MS) was used for sequential peptide and glycan mapping of mAbs without manual intervention. The Agilent AdvanceBio Peptide Plus and Glycan Mapping columns, the latter in combination with the InstantPC kit that allows rapid glycan release and labeling, provide highly informative and precise data. This setup represents a welcome addition to the biopharmaceutical characterization toolbox.

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