Intact analysis of a multi-specific antibody therapeutic

Featuring the X500B QTOF system and intact protein analysis workflows in Biologics Explorer software

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This technical note describes the intact mass analysis of a tri-specific monoclonal antibody (mAb) with various post-translational modifications (PTMs) using 2 new streamlined workflows in Biologics Explorer software. In addition to providing highly accurate and reproducible qualitative and quantitative analysis, the automated deconvolution and time-resolved deconvolution (TRD) workflows offer different deconvolution options and data visualization tools. The workflows were designed to suit diverse applications, from in-depth characterization to routine monitoring.

Tri-specific mAbs are a novel class of genetically modified immunotherapeutics that offer enhanced potency and efficacy compared to conventional modalities. For instance, by targeting multiple antigens, tri-specific mAbs simultaneously block more than one pathways with unique or overlapping functions in pathogenesis[1-2]. In comparison to monospecific mAbs, tri-specific mAbs are structurally more heterogenous with versatile PTMs[1-2]. Therefore, powerful and robust post-acquisition data processing tools are to allow for critical quality attributes (CQAs) monitoring.

In this technical note, the intact and subunit analysis of a tri-specific mAb were achieved using the automated deconvolution and TRD workflows in Biologics Explorer software, Figure 1. The effectiveness of the different deconvolution algorithms toward specific applications and bioanalytical challenges such as complex chromatography are demonstrated.

Key features for the characterization of tri-specific antibodies

- **User-friendly data analysis software:** Biologics Explorer software offers pre-defined parameters for both automated deconvolution and TRD workflows
- **Highly confident identification of co-eluting protein species:** The TRD workflow offers scan by scan deconvolution, which provides advanced traceability to raw MS data and UV chromatography
- **Advanced tools for identifying and monitoring CQAs**: Reliable processing, visualization, reviewing and reporting tools offer an easy path for decision making in biopharmaceutical development

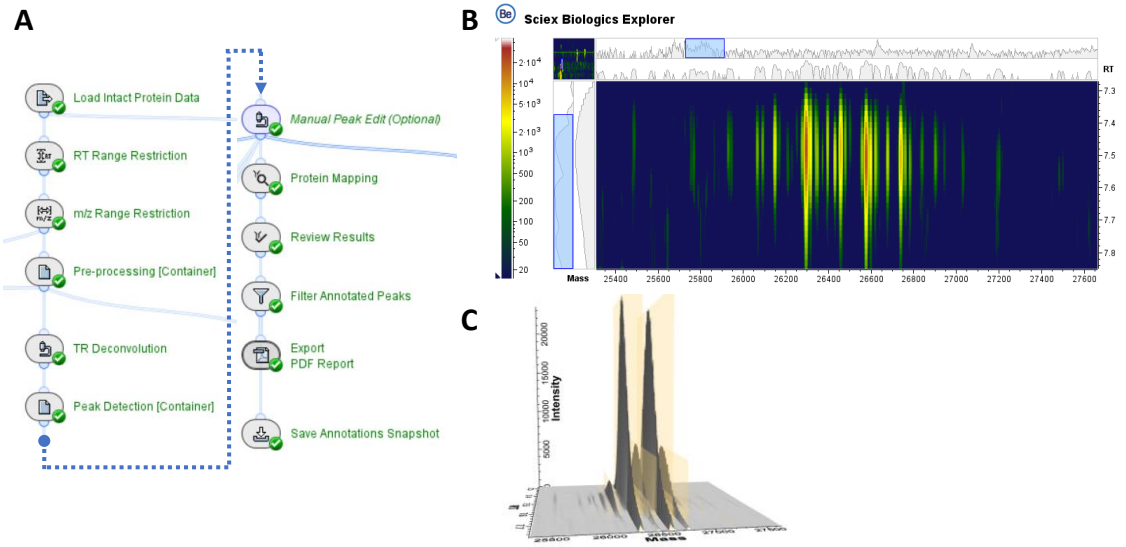

Figure 1. Time-resolved deconvolution of co-eluting protein species. An overview of the streamlined TRD workflow in Biologics Explorer software (A). The reconstructed ion map (B) and the 3D view (C) from the TRD workflow show complex co-eluting species at RT = 7.5 min, including the~~namely,~~ Fc/2 (1) and Fc/2 (2) subunits of the tri-specific mAb.

Methods

Sample preparation: For intact analysis, the tri-specific mAb sample was diluted to 1 mg/mL and subjected to LC-MS analysis. For subunit analysis, site-specific digestion was performed above the hinge region with IgdE (FabALACTICA, GenovisAB). For digestion, 1 unit of enzyme per µg of mAb was added and incubated at 37°C overnight. The IgdE-digested mAbs were denatured and reduced with 30mM dithiothreitol (DTT) with 5M guanidine hydrochloride for 30 min at 37°C.

Chromatography: Separation was accomplished using an ExionLC system (SCIEX) fitted with a Waters BEH SEC column (4.6 mm × 300 mm, 1.7 µm) at 60°C using the gradient shown in Table 1. Mobile phase A was 25mM ammonium formate in water with 0.1% formic acid and mobile phase B was 100% acetonitrile. The flow rate was set to 300 µL/min.

Table 1. LC conditions for intact and subunit analysis.

Time (min)	%A	%B
Initial	*30*	*70*
10.0	*30*	*70*
10.1	*25*	*75*
20.0	*25*	*75*
20.1	*30*	*70*
35.0	*30*	*70*

Mass spectrometry: A SCIEX X500B QTOF system was used for data acquisition. Data were acquired using TOF MS mode with the intact protein mode turned on. Detailed MS parameters are listed in Table 2.

Data processing: Data were processed with Biologics Explorer software. Raw data (.wiff2) collected with the X500B QTOF system were directly loaded into the workflows. Intact mass analysis was conducted in the automated deconvolution workflow and the TRD workflow was selected for subunit analysis.

Table 2. MS parameters for intact and subunit analysis.

Parameter	Setting
Scan mode	*TOF MS Positive*
Ion Spray Voltage	*5000V*
Gas 1	*50 psi*
Gas 2	*50 psi*
Curtain gas	*30 psi / 35 psi (IgdE reduced)*
Ion source temperature	*300°C / 450°C (IgdE reduced)*
Time Bins to Sum	*80*
Accumulation time	*0.5 sec*
Mass range	*600 - 5,000 m/z*
Declustering potential	*300 V (intact)/ 150 V (subunit)*
CAD gas	*7*
Collision energy	*10 V*

Overview of the tri-specific mAb

The tri-specific mAb consists of 4 chains: LC1, LC2, HC1 and HC2 (Figure 2). LC1 and HC1 were bioengin

ered with an additional antigen binding domain, increasing the heterogeneity of the protein. The mAb contains 3 consensus

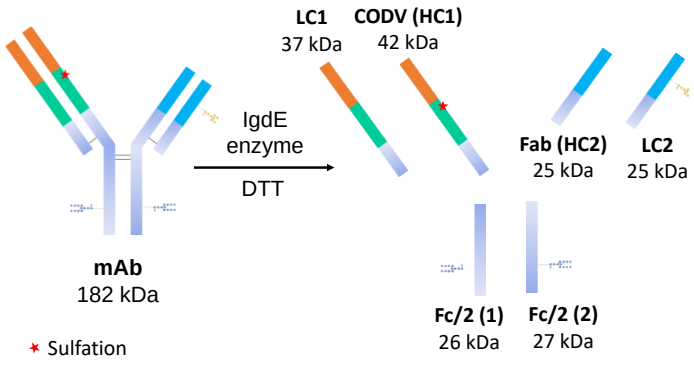

Figure 2. Sample preparation for subunit mass analysis via site-specific digestion using IgdE followed by reduction.

sequences with N-linked glycosylations (2 on the heavy chains and 1 on LC2), which increases the complexity further. In addition, the HC1 contains a sulfation site, indicated by an asterisk in Figure 2.

Intact analysis of the tri-specific mAb using the automated deconvolution workflow

The automated deconvolution workflow was designed to be a fast data interpretation and monitoring tool for intact mass analysis. The automated deconvolution workflow involves 2 steps. First, retention time (RT) ranges are determined either automatically by software or manually set by the user (Figure 3A). Then, all scans within each defined RT range are summed, followed by spectrum deconvolution. All default parameters are pre-optimized, allowing this workflow to offer a fast processing speed. Therefore, users can get answers with a short turn-around time, which provides a potential for high throughput analysis in drug screening and routine monitoring.

A comprehensive characterization was performed to fully evaluate the molecular integrity and composition of the tri-specific mAb. The molecule was analyzed in its glycosylated intact state. The raw data was inspected with an ion map (Figure 3A) and raw TOF MS spectra (Figure 3B), and the reconstructed spectra (Figure 3C) were analyzed using the theoretical mass information (Table 3).

The observed mass of the mAb was 80 Da higher than the expected molecular weight based on the sequence, indicating the existence of either 1 phosphorylation or 1 sulfation. Additional subunit analysis (see next section), peptide mapping experiments and binding assays revealed that this tri-specific mAb contains 1 sulfation on HC1, which affected its binding to the antigen (data not shown). As seen in Figure 3C, multiple glycoforms were detected for the tri-specific mAb, which highlights its increased complexity. Since different glycan combinations can result in the same mass shift on the intact level, the glycosylation profile was further verified with subunit analysis.

Table 3. Summary of glycosylated intact mass analysis.

Proteoform	Calculated mass [Da]	Experimental mass [Da]	Delta mass [Da]
2*G0F + G2S1 + sulfation	181779.9	181783.3	3.4
G0F + G1F + G2S2 + sulfation	182233.3	182235.6	2.3
2*G1F + G2S2 + sulfation	182395.4	182396.2	0.8
G1F + G2F + G2S2 + sulfation	182557.6	182557.3	-0.3

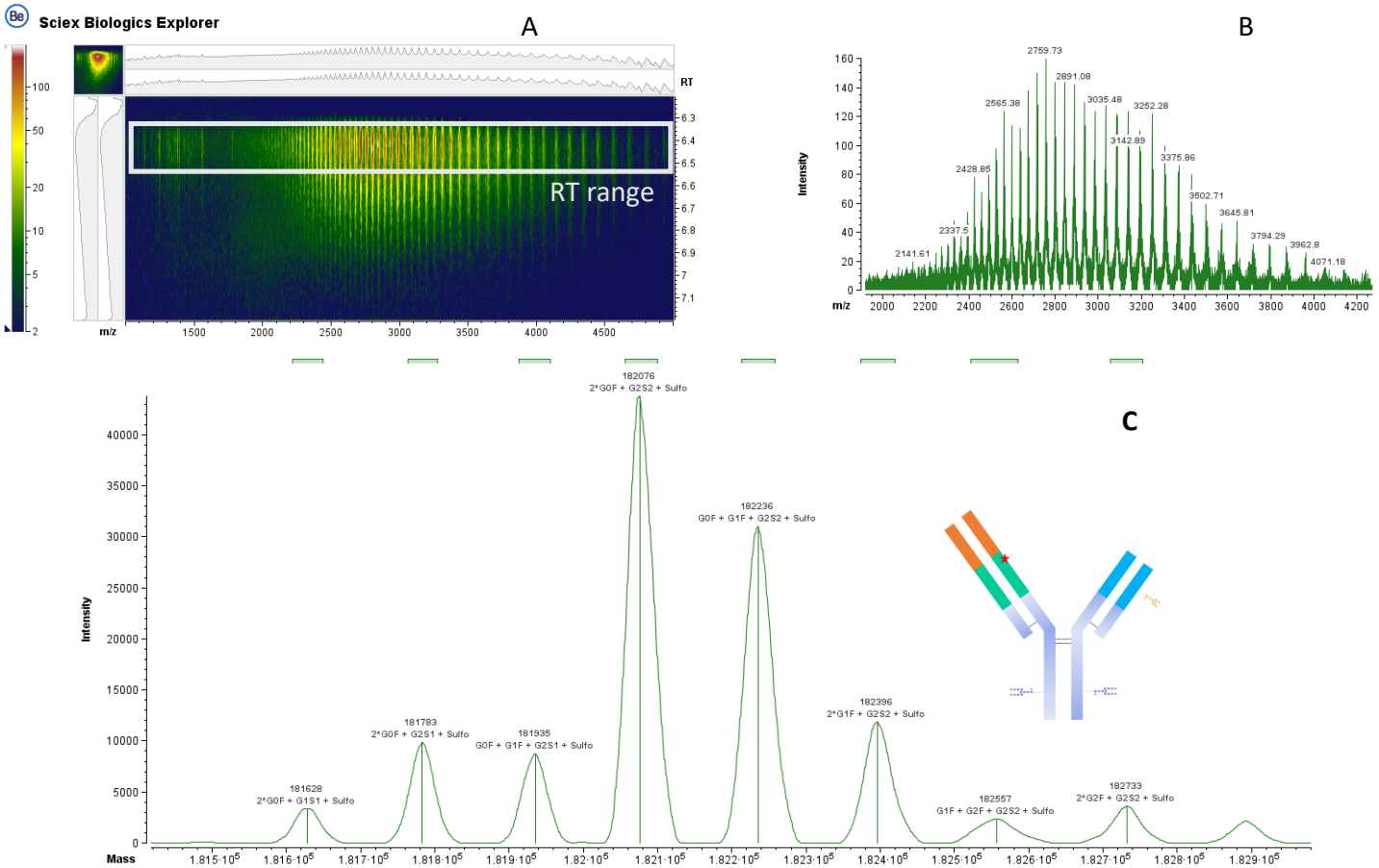

Figure 3. Raw and reconstructed spectra of the intact tri-specific mAb. Ion map showing protein envelop distribution from RT = 6.3 to 7.1 min (A), raw MS spectrum at RT = 6.4 min (B), and reconstructed spectrum showing multiple glycoforms of the tri-specific mAb (C).

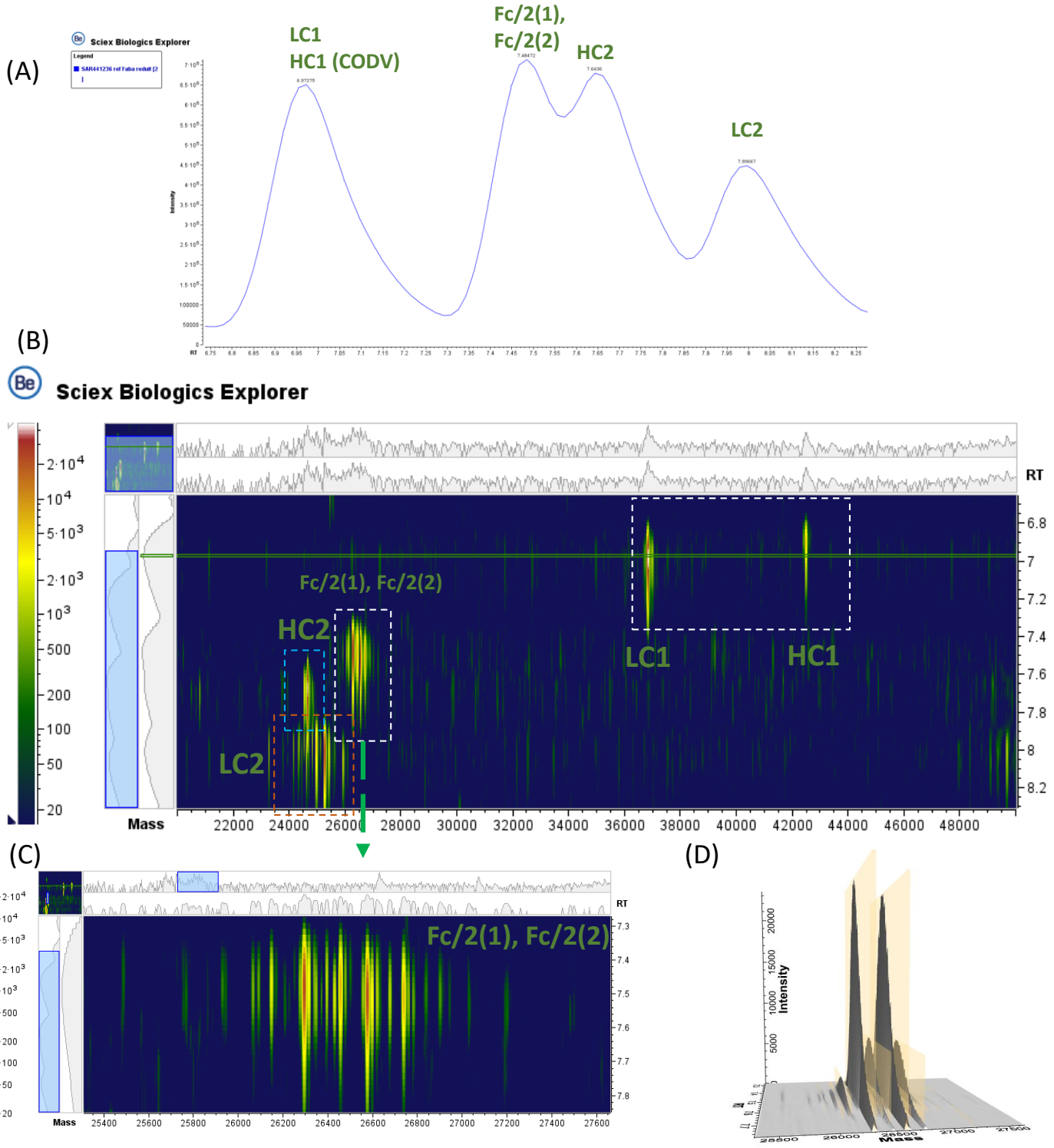

Figure 4. Subunit analysis of the tri-specific mAb using the TRD workflow. Total ion chromatogram (TIC) showing the presence co-eluting subunits (A), reconstructed ion map (B), zoomed-in view of species at RT = 7.5 min (C) and 3D view of the co-eluting Fc/2 subunits (D).

Subunit analysis of the tri-specific mAb using TRD workflow

The TRD algorithm offers scan-by-scan spectrum deconvolution, which enables the detection of low abundant species. The reconstructed data from each scan are used to generate an ion map or 3D views for excellent visualization of the results. Therefore, it benefits the characterization of complex samples, especially those containing co-eluting species, for which in-depth characterization and maximum visibility to sample constituents are crucial.

In subunit analysis, the tri-specific mAb was digested using IgdE followed by reduction, resulting in 6 different subunits (Figure 2). Despite the incomplete chromatographic separation of these subunits (Figure 4A), the TRD workflow was employed to successfully identify all the subunits. Figure 4B shows the ion map of reconstructed mass spectra produced from TRD, allowing exceptional visibility to co-eluting protein species. From the ion map2 co-eluting species were detected at RT = 6.9 min (Figure 4B), which were identified as LC1 and Fab HC1. A zoomed-in view of the ion map (Figure 4C) and 3D view (Figure 4D) revealed the existence of multiple species at RT = 7.5 min.

The TRD algorithm allows for the accurate identification of each subunit (Figure 5). The LC1 (observed mass at 36832.6 Da) and Fab (HC2) (observed mass at 24669.7 Da) subunits were identified with excellent mass accuracy that was lower than 0.1Da (Figure 5 and Table 4). The Fab HC1 (CODV) subunits with or without sulfation were identified, with the sulfated species being the major form detected at 42485.8 Da (Figure 5 and Table 4). For the LC2 subunit, 10 N-linked glycoforms were detected, and most of them were sialylated. G2S2, G2S1, G3S3, and G2FS2 are the major glycan species observed. Despite the co-elution between Fc/2 (1) and Fc/2 (2), an accurate identification was achieved, indicating G0F and G1F as the predominant glycoforms for both subunits. The results were consistent with intact mass analysis. As such TRD is the optimal solution for analyzing samples with complex chromatography.

In summary, the automated deconvolution and TRD workflows in the Biologics Explorer software allowed for fast analysis and comprehensive characterization of the tri-specific mAbs. The streamlined workflows provided information about the integrity of the mAb and revealed domain-specific information, including different modifications. Intact mass and subunit analysis provided sequence confirmation, identification of critical modifications and detailed glycan profiling for each domain.

Table 4. Summary of the species detected in subunit analysis of the tri-specific mAb

Proteoform	Calc. mass [Da]	Experimental mass [Da]	Delta mass [Da]
LC1(CODV)	36832.5	36832.6	0.11
Fab(HC CODV), sulfation	42486.0	42485.8	-0.20
Fab(HC CODV)	42405.9	42406.1	0.12
HC1 (CODV)[1-387], sulfation	42384.9	42385.1	0.17
LC2+G1S1	24834.0	24833.1	-0.85
LC2+G0	24380.6	24380.6	-0.04
LC2+G2FS1	25142.3	25141.9	-0.37
LC2+G2S1	24996.1	24995.9	-0.24
LC2+G2S1	24978.1	24977.6	-0.54
LC2+G3S2	25652.7	25652.6	-0.17
LC2+G2FS2	25433.5	25433.0	-0.49
LC2+G2S2	25287.4	25287.1	-0.27
LC2+G2S2	25349.4	25348.7	-0.75
LC2+G3S3	25944.0	25943.6	-0.40
Fab(HC2)	24669.8	24669.7	-0.07
HC2[1-226]	24568.7	24568.5	-0.11
Fc(HC2)+G0F-GlcNAc	26092.1	26091.9	-0.22
Fc(HC2)+G0	26149.1	26149.0	-0.15
Fc(HC2)+G1F	26457.4	26457.3	-0.14
Fc(HC2)+G0F	26295.3	26295.3	0.00
Fc(HC2)+G2F	26619.5	26620.1	0.52
HC2[227-450]+G0F	26396.4	26396.0	-0.41
Fc(HC CODV)+G2F	26900.9	26901.5	0.65
Fc(HC CODV)+G0F-GlcNAc	26373.4	26372.6	-0.77
Fc(HC CODV)+G0	26430.5	26430.4	-0.08
Fc(HC CODV)+G1F	26738.8	26738.8	0.01
Fc(HC CODV)+G0F	26576.6	26576.6	-0.06
HC1 (CODV)[388-611]+G0F	26677.7	26677.2	-0.56

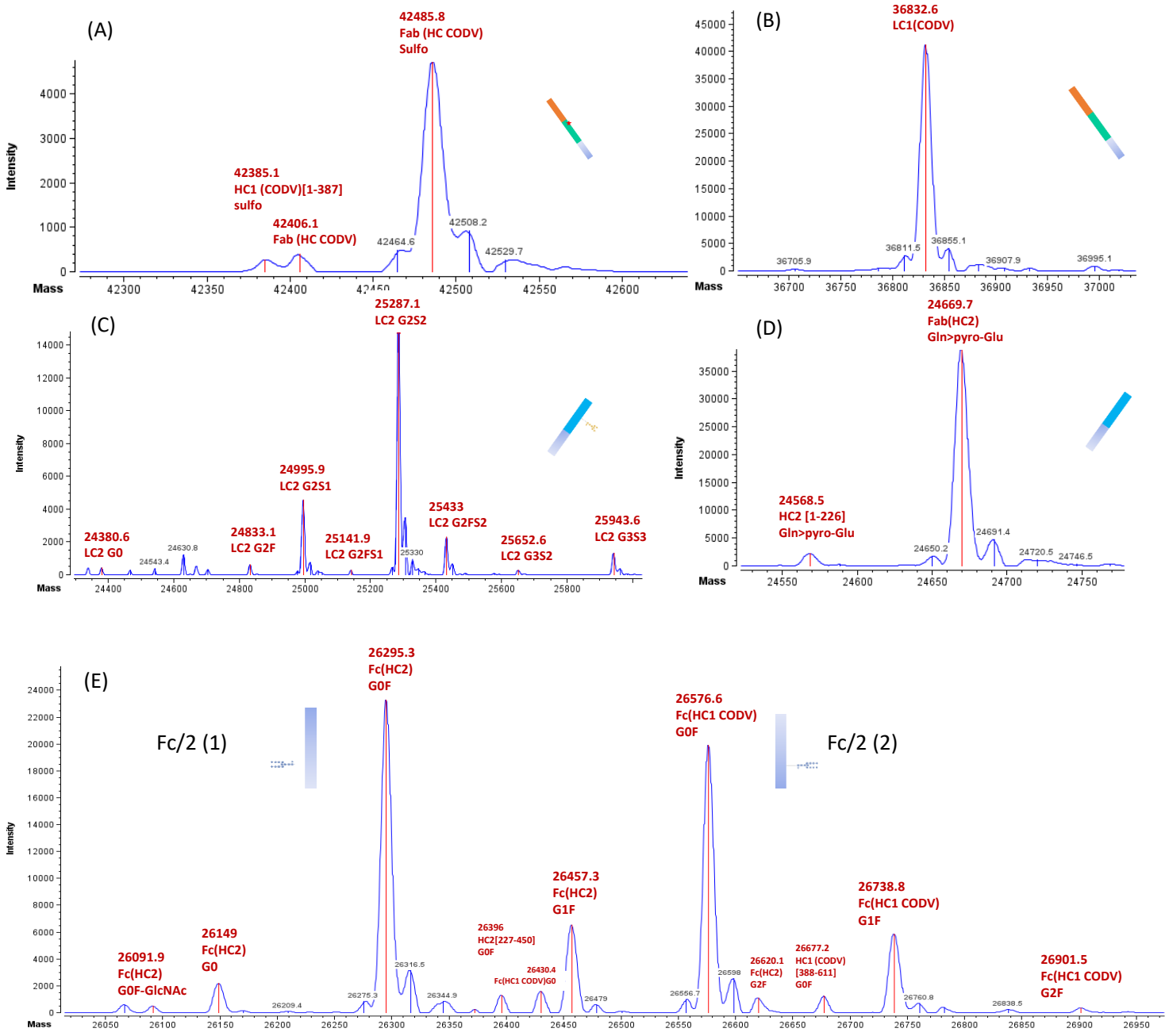

Figure 5. Reconstructed and annotated spectra of the tri-specific mAb subunits. (A) HC1 (CODV) showed sulfation and was the major species observed. (B) LC1 and (C) LC2 showed heavily sialylated glycan profiles. (D) HC2, (E) Fc/2(1) and Fc/2 (2) showed that G0F, G1F and G2F were the major glycans observed in the Fc domain.

Conclusions

- Two complementary workflows offer a streamlined approach for comprehensive intact mass/subunit analysis of next-generation biologics
- The automated deconvolution workflow is a powerful platform for mAb screening and routine monitoring where quick analysis is desired.
- Given the superior scan-by-scan TRD algorithm, accurate identification of co-eluting protein species can be efficiently achieved
- The excellent data quality from the X500B QTOF system and its direct compatibility with Biologics Explorer software offer a generic and time-efficient platform for the characterization of complex next-generation biotherapeutics

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