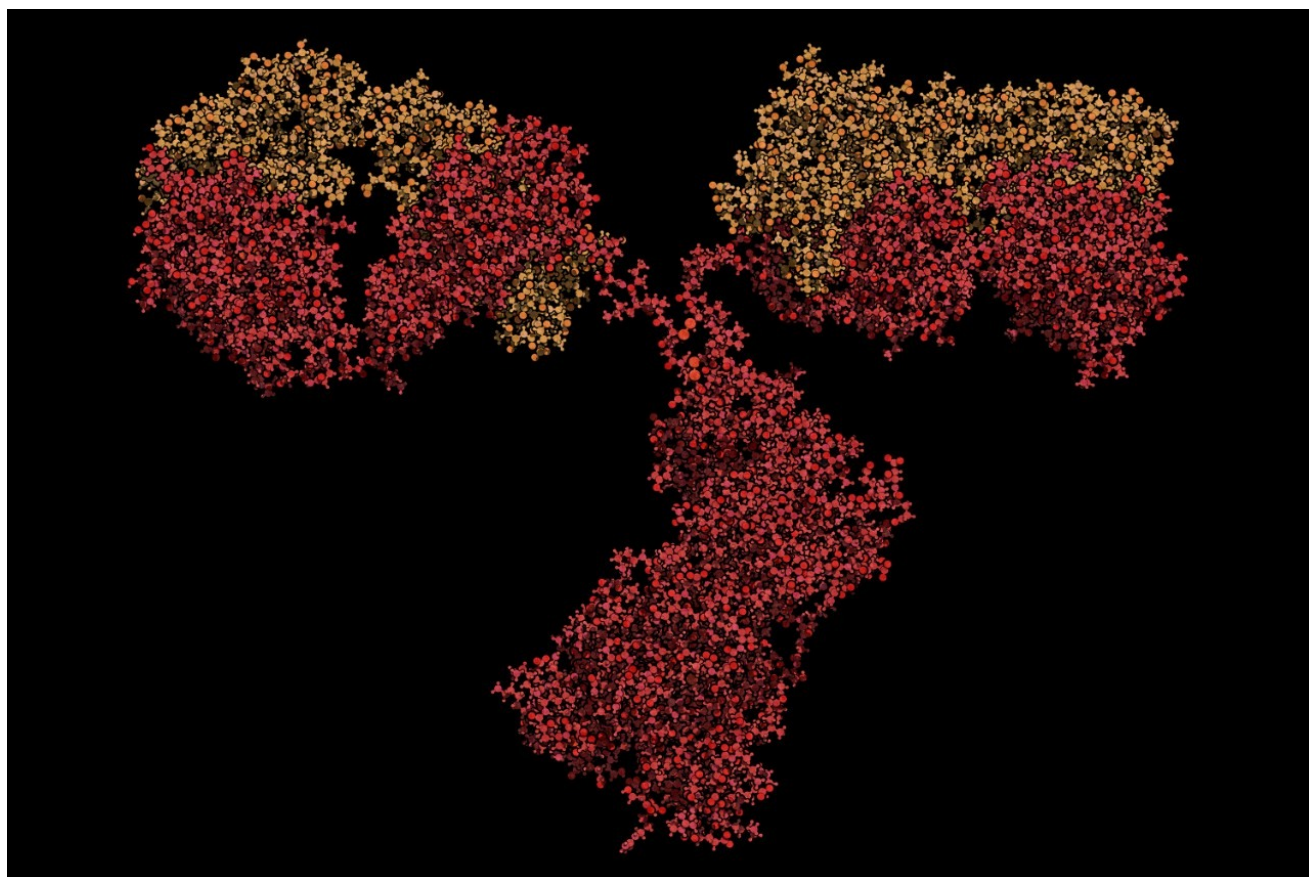


Top-down Monoclonal Antibody (mAb) Analysis Using CID and ETD Fragmentation and the UNIFI Scientific Information System

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Abstract

This application note demonstrates the acquisition of protein top-down analysis data by collision induced dissociation (CID) or electron transfer dissociation (ETD) MS-MS fragmentation from the SYNAPT G2-Si HDMS Mass Spectrometer.

Benefits

The UNIFI Scientific Information System streamlines data processing, reviewing, and reporting for monoclonal antibody fragmentation data, and enables the confirmation of protein sequences and post-translational modifications.

Introduction

Direct fragmentation of proteins (top-down) provides an orthogonal approach to verify terminal sequence confirmation and localize protein modifications. Protein fragmentation approaches are primarily qualitative and have limited dynamic range to assess variation. Additionally, these approaches have no simple mechanism to determine modification abundance, making them a complementary technology to peptide mapping for mAbs and other biotherapeutics. Top-down protein fragmentation generates diverse fragment ion types and a multitude of charge states with overlapping spectral patterns. This data complexity complicates the process of producing primary structural assignments – usually through manual annotation – leading to the necessary development of software tools that automatically deconvolute raw spectral MS-MS data, annotate fragmentation patterns to protein sequences, and generate reports. These challenges have impeded broader usage of top-down protein analysis for routine biotherapeutic mAb development.

This application note demonstrates the acquisition of protein top-down analysis data by collision induced dissociation (CID) or electron transfer dissociation (ETD) MS-MS fragmentation from the SYNAPT G2-Si HDMS Mass Spectrometer (Figure 1). The data from these analyses was imported into UNIFI for processing, review, and report generation. This automated data processing workflow enabled efficient sequence verification, along with identification of modifications for a mAb and its subunits.

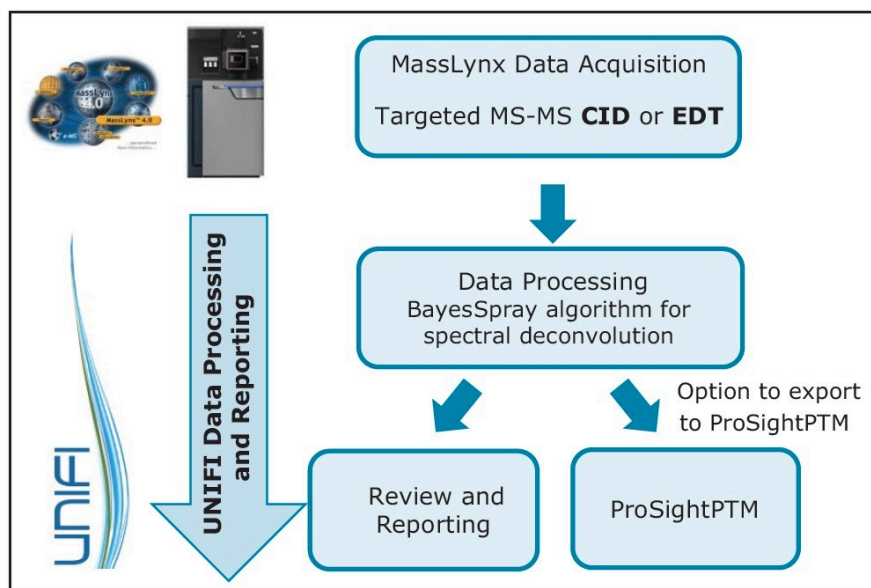


Figure 1. UNIFI top-down analysis workflow automates processing and reporting for targeted MS-MS data acquired using MassLynx.

Experimental

Methods

Trastuzumab was treated by IdeS (Genovis) protease and reduced by DTT (SigmaAldrich) to produce light chain (LC), Fd, and Fc/2 fragments. Intact mass and the subsequent top-down analyses were performed on an ACQUITY UPLC H-Class Bio System connected to a SYNAPT G2-Si HDMS Mass Spectrometer. The analyses were processed in UNIFI using the dedicated protein intact mass and top-down workflows. Data acquisition for the CID or ETD MS-MS fragmentation was performed on selected charge states.

LC conditions

LC system:	ACQUITY UPLC H-Class Bio System
Detector:	ACQUITY UPLC Tunable UV (TUV), 280 nm
Column:	ACQUITY UPLC Protein BEH C ₄ , 1.7 µm, 2.1 mm x 50 mm (P/N 186004495)

Column temp.:	80 °C
Sample temp.:	4 °C
Mobile phase A:	0.1% formic acid
Mobile phase B:	0.1% formic acid in acetonitrile

Gradient table:

Time(min)	Flow rate (mL/min)	%A	%B	Curve
Initial	0.4	95	5	Initial
1.00	0.4	95	5	6
1.10	0.1	95	5	6
2.50	0.1	77	23	6
12.50	0.1	71	29	6
13.00	0.1	5	95	6
13.10	0.4	5	95	6
14.00	0.4	5	95	6
14.50	0.4	95	5	6
17.00	0.4	95	5	6

Targeted MS-MS analysis

MS system:	SYNAPT G2-Si HDMS
Mode:	ESI+ sensitivity mode
Capillary:	2 kV
Sample cone voltage:	40 V
Source temp.:	120 °C
Desolvation temp.:	500 °C
Desolvation gas flow:	800 L/h
Full scan MS:	Scan rate=0.5 sec Mass range=500–2000 <i>m/z</i>

Targeted MS-MS conditions

Scan rate=1 Hz; mass range=50–2000 *m/z*

For CID fragmentation, the collision energy level was ramped from 20 to 40 eV

For ETD fragmentation, MS-MS data was acquired over a one second scan (signal accumulation) period with an anion refill time of 100 ms between scans

Informatics

MassLynx v4.1 for SYNAPT instrument control and data acquisition

UNIFI Scientific Information System v1.8 for data processing, reviewing, and reporting¹

ProSight PTM 2.0 as an alternative informatics annotation engine using data exported from UNIFI in the .PUF file format²

Results and Discussion

Intact mass analysis of trastuzumab subunit

Trastuzumab subunit LC-MS analysis data was acquired on an ACQUITY UPLC H-Class Bio System coupled with a SYNAPT G2-Si HDMS Mass Spectrometer under MassLynx control. The data was then imported into UNIFI for processing, review, and report generation. The UNIFI review panel (Figure 2) includes: (A) A component summary table displaying the trastuzumab subunit and assigned glycoforms; (B) Total ion count (TIC) and base peak ion (BPI) chromatograms; (C) Summed raw MS spectra and MaxEnt1 deconvoluted spectra; (D) Component plot.

The T-mab subunits and their modified forms were identified. The light chain (LC), Fd, and Fc/2 glycoforms (G0, G0F, G1F, and G2F) were displayed with the deconvoluted average mass in the component table (Figure 2A) and the component plot (Figure 2D). The TIC chromatogram exhibited good separation of the subunit peaks with identity annotation (Figure 2B). Easy access to the raw and deconvoluted spectra facilitated data inspection and reviewing (Figure 2C).

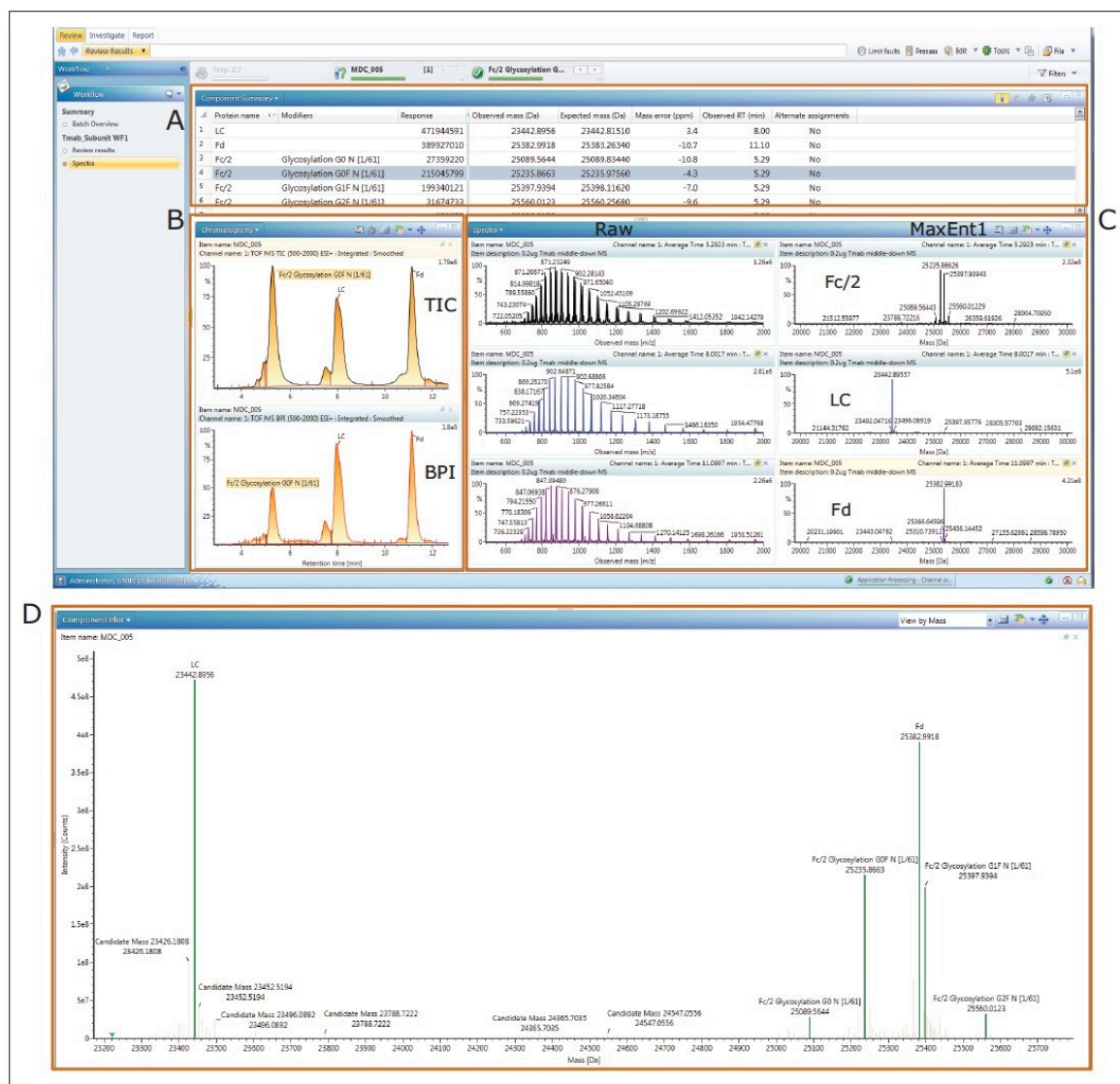


Figure 2. Trastuzumab (T-mab) subunit LC-MS analysis with MaxEnt1 deconvolution processing and UNIFI intact MS analysis workflow.

Top-down analysis of trastuzumab subunit with ETD fragmentation

Following the subunit analysis, a highly-charged precursor ion was chosen for efficient ETD fragmentation with automated data processing and reporting.

UNIFI provides a new protein top-down analysis workflow (Figure 3) featuring a novel Bayesian inference algorithm – BayesSpray – for deconvolution of both isotope resolved (peptide) and non-resolved (protein) data. It can automate the processing and reporting of collision induced dissociation (CID) as well as electron transfer dissociation (ETD) MS-MS fragmentation, enabling streamlined sequence verification and identification of modification sites.

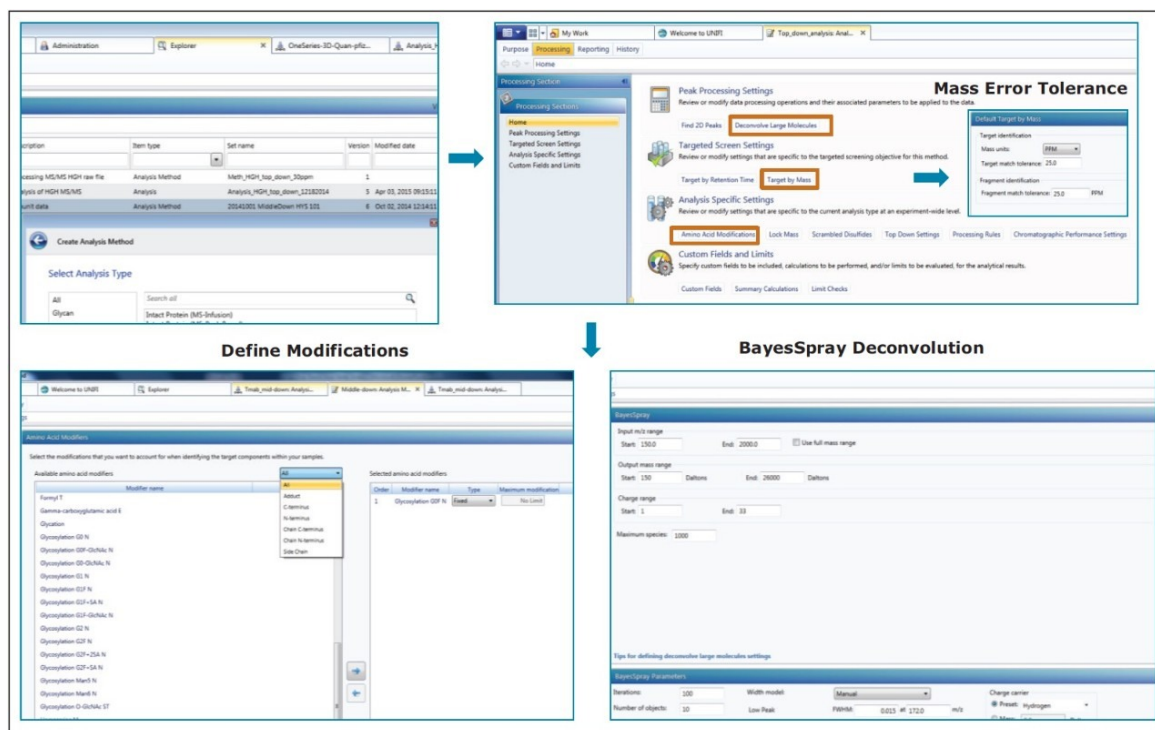


Figure 3. Create analysis method for top-down analysis.

An example data of infusion top-down analysis with ETD fragmentation on Fc/2 subunit is shown in Figure 4.

One precursor ion (765.8 m/z , 33+) corresponding to the Fc/2 G0F glycoform was selected for ETD fragmentation. The MassLynx raw data was then imported into UNIFI for automated processing. The review panel display can be easily customized and configured to interactively investigate the processed data. As an example, an intuitive data review panel is displayed in Figure 4: (A) Component summary table to display the identified trastuzumab subunit; (B) Sequence coverage map; (C) Summed raw, BayesSpray mocked, and deconvoluted CID fragmentation spectra; (D) Annotated deconvoluted spectra and fragmentation ion table. 42 c- and 41 z-ions were assigned, which is roughly 38.1% backbone fragment ion coverage for the Fc/2 subunit.

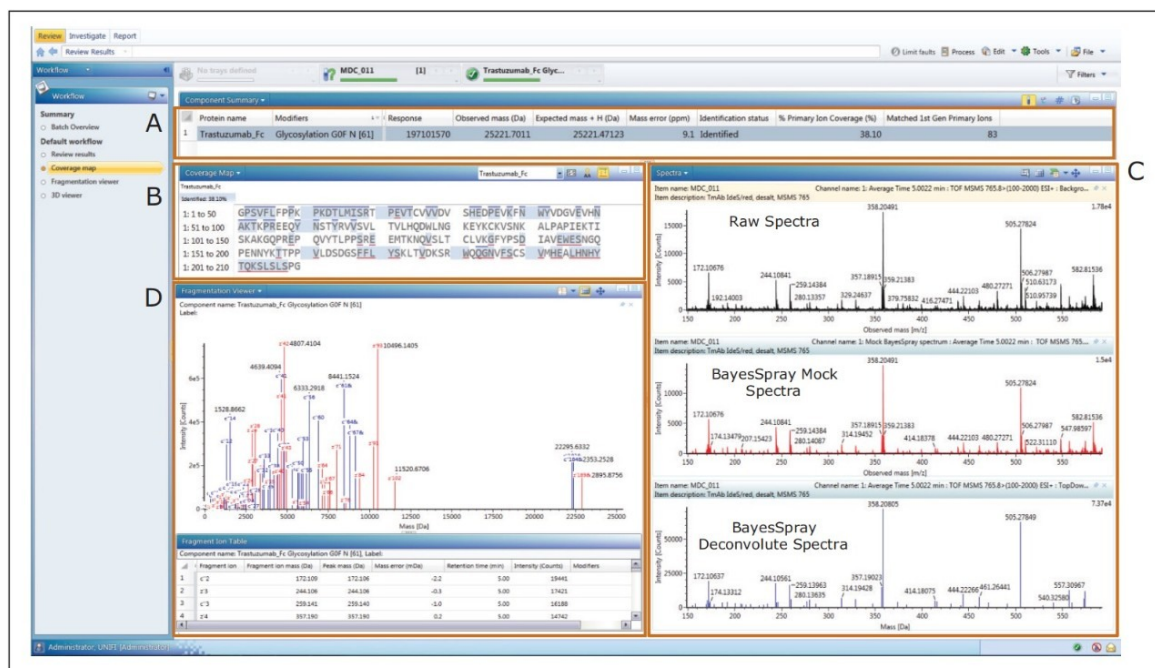


Figure 4. Example of infusion top-down analysis of the trastuzumab Fc/2 subunits with targeted MS-MS data acquisition using ETD fragmentation.

The UNIFI top-down workflow offers an option to export the processed MS-MS data (BayesSpray deconvoluted fragment ions/intensity list) in a .PUF format file that can be processed further with ProSight. ProSight was designed by the Proteomics Center of Excellence at Northeastern University for protein top-down analysis in order to identify fragments and obtain sequence coverage information.

The example result from ProSight PTM 2.0 assigned the same fragment ions and had the same backbone fragment ion coverage as the UNIFI results (Figure 5). However, the amino acid modifications must be assigned manually to be considered when using ProSight, whereas a UNIFI analysis method can include multiple modifications. In this case, ProSight PTM highlighted N in orange, which was assigned with a custom modification by manually adding the mass of GOF.

Cysteine Custom										
ID/Gene	Length	Mass	Mass Diff.	PPM Diff.	C Ions	Z Ions	Total Ions	PDE Score	Expectation	P Score
User Defined Protein. (Type: basic, Signal Peptide: false, Propep: false)										
c1	-G-P-T-S-V-T-F-L-T-F-P-P-K-P-K-D-T-L-M-I-S-R-T-P-E-T-V-T-C-V-V-V-D-V-	z181								
c31	-S-H-E-T-D-P-E-T-V-K-F-N-W-Y-V-D-G-V-E-V-H-N-A-T-K-T-T-K-P-R-T-E-E-Q-Y-	z151								
c61	-N-T-S-T-Y-T-R-V-V-T-S-V-L-T-V-L-H-Q-D-W-L-N-G-K-E-Y-K-C-K-V-S-N-K-	z121								
c91	-A-L-P-A-P-I-E-K-T-I-S-K-A-K-G-Q-P-R-E-P-Q-V-Y-T-L-P-P-T-S-R-E-	z91								
c121	-E-M-T-K-N-Q-L-V-S-L-T-L-L-V-K-G-F-Y-P-S-D-I-A-V-E-T-W-E-T-S-N-G-Q-	z61								
c151	-P-E-N-N-Y-K-T-T-P-P-L-V-L-D-S-D-G-S-F-F-L-L-Y-S-K-L-T-L-V-D-K-S-R-	z31								
c181	-W-Q-T-Q-G-T-N-V-L-F-T-S-C-S-L-V-M-H-E-A-L-L-H-N-H-Y-T-T-Q-K-T-S-L-T-S-L-T-S-P-G-	z1								
ID/Gene	Length	Mass	Mass Diff.	PPM Diff.	C Ions	Z Ions	Total Ions	PDE Score	Expectation	P Score
0	210	25220.5000	0.2396	9.5015	42	41	83	9.45	2.54e-33	2.54e-33
Take to Sequence Gazer									RESID	SEQ

Figure 5. ProSight PTM2.0 analysis results of the previously described experiment provide the same backbone fragment ion coverage as UNIFI.

Top-down CID fragmentation followed by IMS separation

Beyond the conventional top-down methodology, the SYNAPT G2-Si HDMS Mass Spectrometer provides a unique capability for postfragmentation ion mobility separation. This additional gas phase separation of the fragment ions is based on the charge and collision cross sectional differences (Figure 6).

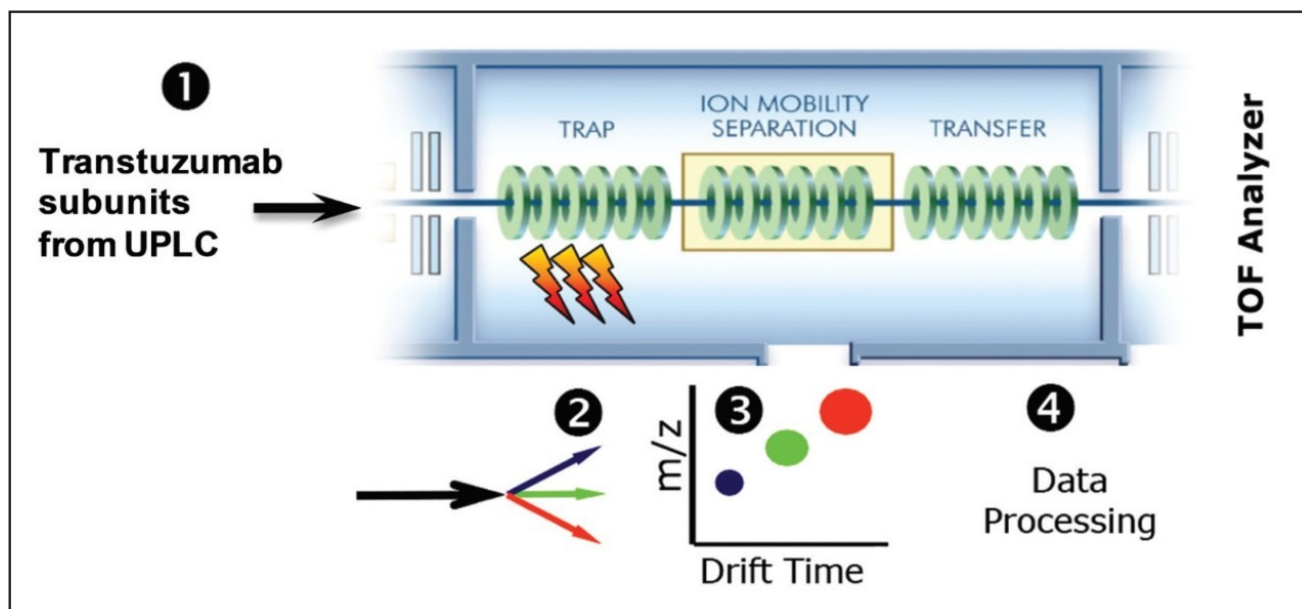


Figure 6. Schematic of ion mobility-based fragment separations provided by SYNAPT G2-Si HDMS. (1) Select one charge state of the trastuzumab subunit using the quadrupole. (2) Fragment using CID or ETD in the TRAP cell. (3) Separate CID fragment ions based on charge, size, and mass by ion mobility. (4) Process to simplify the fragmentation data.

The same sample was separately analyzed by LC-MS/MS (CID-IMS) on SYNAPT G2-Si HDMS. Singly and multiply charged fragment ions were extracted into separate raw files by DriftScope prior to the data being imported into UNIFI and processed. Figure 7 shows the Fc/2 G0F (precursor m/z 902.2, 28+) top-down analysis data with CID fragmentation followed by ion mobility separation. Figure 7B and 7C display the results derived from isolated, singly charged and multiply charged fragments. The raw spectra with only the 1+ charged ions facilitated the manual data interpretation. The fragments were assigned exclusively to the N- and C- terminal sequences. In total, 19 b- and 31 y-ions were assigned, which is roughly 23.81% fragment ion coverage for the Fc/2 subunit. This additional separation produced more intuitive spectra, and simplified data review and terminal sequence confirmation.

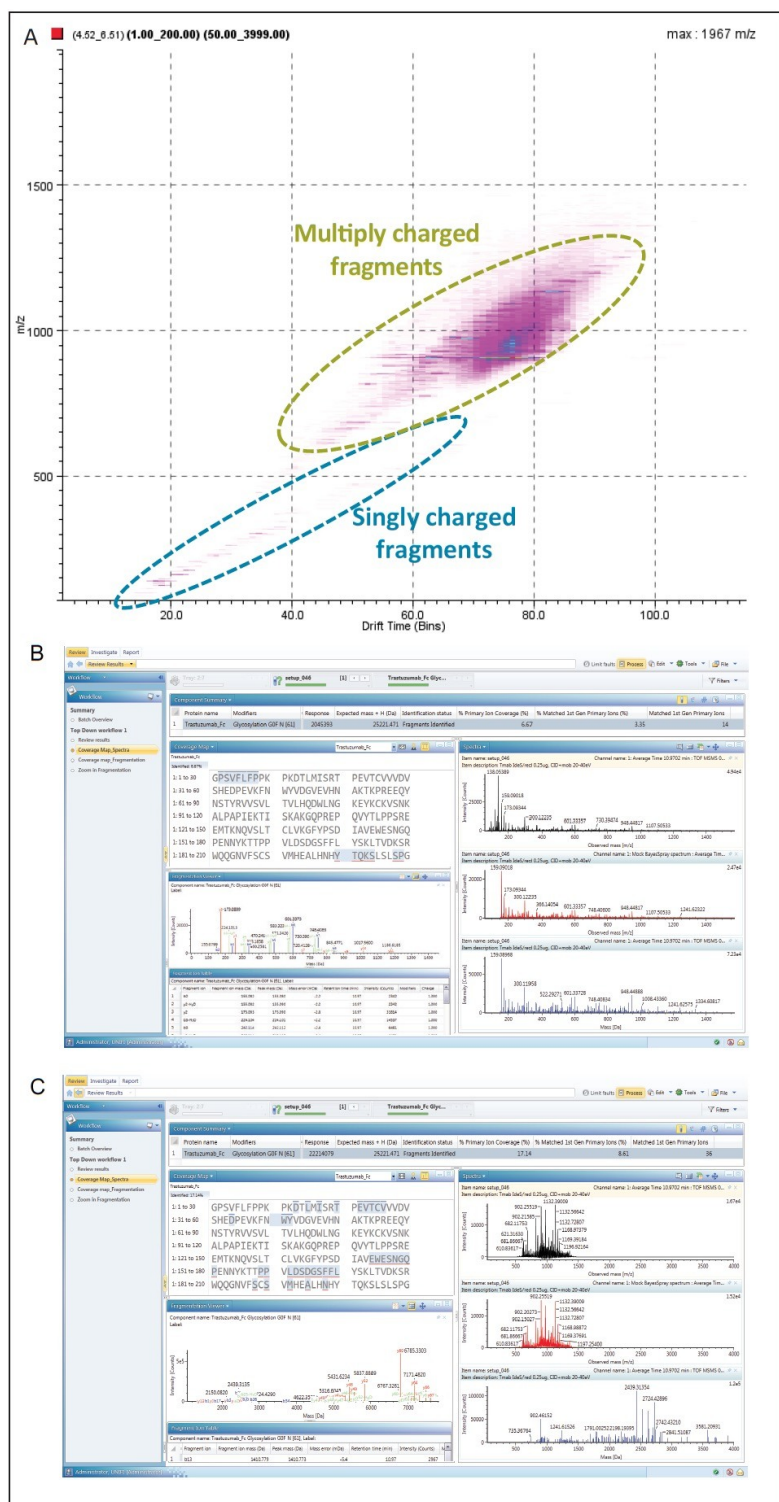


Figure 7. Example of LC-MS/MS top-down

analysis of the trastuzumab Fc/2 subunits with targeted MS-MS data acquisition using CID fragmentation

Report generation of top-down analysis

followed by ion mobility fragment separation. (A) Extracted ions using DriftScope. (B) Review panel of multiply charged fragment ions data processing result. (C) Review panel of singly charged fragment ions data processing

Report templates can be created and customized to meet specific requirements, and then saved to be applied to result.

future analyses. Figure 8 provides a snapshot of the trastuzumab Fc/2 CID-IMS top-down analysis report of extracted singly and multiply charged fragment ions. This report includes acquisition and sample information, sequence coverage map, fragmentation viewer/table, and 3D illustration of IMS data – all of which can be organized within a single report format. The object properties and report templates are user-configurable. Multiple reports can be executed for one analysis to answer all scientific questions in an efficient format.

Analysis Information

Analysis Information

Item name: Tmab_CID+IMS_120315 Analysis Method Item Middle-down
name:
Version: 1 Analysis Method Version: 1
Modified date: Dec 03, 2015 10:39:25 Eastern Standard Time Sample Set Created date:
Modified by: Administrator, UNIFI Sample Set Instrument
system name:
Folder: Company/TopDown/Martin DeCecco

Summary Table

Item names: setup_046, Sample position: 27, Replicate number: 1

Protein name	Modifiers	% Primary Ion Coverage (%)	Matched 1st Gen Primary Ions	Instrument system name
1 Trastuzumab_Fc	Glycosylation GOF N [61]	6.67	14	SYNAPTGS-Si#NorSet

Singly Charged Fragments

Item names: setup_046, Sample position: 27, Replicate number: 1

Protein name	Modifiers	% Primary Ion Coverage (%)	Matched 1st Gen Primary Ions	Instrument system name
1 Trastuzumab_Fc	Glycosylation GOF N [61]	17.14	36	SYNAPTGS-Si#NorSet

Multiply Charged Fragments

Coverage Map

Component name: Trastuzumab_Fc Glycosylation GOF N [61]

Trastuzumab_Fc Singly Charged Fragments

Coverage: 6.67%

1: 1 to 80 GPSVFLPPK PKDTLIESRT PEVTCVWDV SHEDPEVKFN WVDGVEVHN AKTKPREQY NSTYRVSVL TVLHQDLNG
1: 81 to 160 KEVKCKVSNK ALPAPLEKTI SKWQGPREP QVYTLPPSR EHTKNQVSLT CLKVGFYPSD IAVENESNGQ PENNYKTTTP
1: 161 to 210 VLSDSGSFLL YSKLTVDKSR MQQGVFSCS VHEALHHY TQKSLSLSPG

Amino acids confirmed by N-terminal fragmentation are overlined.
Amino acids confirmed by C-terminal fragmentation are underlined.

Trastuzumab_Fc Multiply Charged Fragments

Coverage: 17.14%

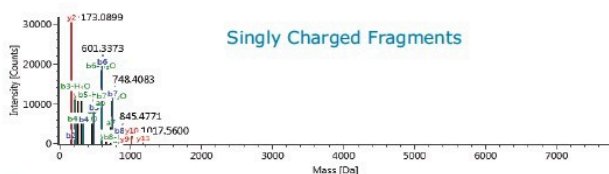
1: 1 to 80 GPSVFLPPK PKDTLIESRT PEVTCVWDV SHEDPEVKFN WVDGVEVHN AKTKPREQY NSTYRVSVL TVLHQDLNG
1: 81 to 160 KEVKCKVSNK ALPAPLEKTI SKWQGPREP QVYTLPPSR EHTKNQVSLT CLKVGFYPSD IAVENESNGQ PENNYKTTTP
1: 161 to 210 VLSDSGSFLL YSKLTVDKSR MQQGVFSCS VHEALHHY TQKSLSLSPG

Amino acids confirmed by N-terminal fragmentation are overlined.
Amino acids confirmed by C-terminal fragmentation are underlined.

Annotated Deconvolute Spectra

Component names: Trastuzumab_Fc Glycosylation GOF N [61]

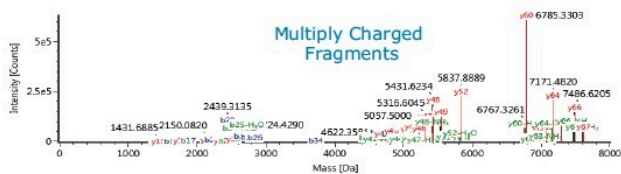
Label:



Singly Charged Fragments

Component name: Trastuzumab_Fc Glycosylation GOF N [61]

Label:



Multiply Charged Fragments

Sample List

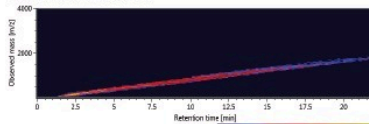
Analysis injection list

Item name	Item description	Sample type	Replicate number	Sample position	Acquisition status
1 setup_046	Tmab CID+IMS 0.25ug, CID+mob 20-40eV	Unknown	1	27	Complete
2 setup_046	Tmab CID+IMS 0.25ug, CID+mob 20-40eV	Unknown	1	27	Complete

3D Viewer (Ion Mobility)

Item names: setup_046

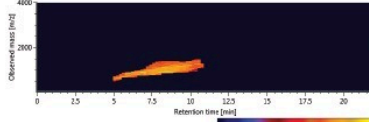
Channel name: 1: TOF MSMS 0- (30-4000) ES+



Singly Charged Fragments

Item names: setup_046

Channel name: 1: TOF MSMS 0- (30-4000) ES+



Multiply Charged Fragments

Assigned Fragment List

Fragment ion	Fragment ion mass (Da)	Peak mass (Da)	Mass error (mDa)	Intensity (Counts)	Modifiers	Charge
b4	341.182	341.183	0.000	6349		1
a5	460.256	460.258	0.002	8346		1
b5-H2O	470.240	470.241	0.001	12531		1
b5	488.251	488.253	0.003	9310		1
a6	573.340	573.342	0.002	10307		1
b6-H2O	583.324	583.323	-0.001	10704		1
b6	601.315	601.317	0.002	20722		1
y7	668.357	668.341	-0.016	1272		1
a7	720.408	720.413	0.004	1406		1
b7-H2O	730.393	730.397	0.004	12151		1
b7	748.403	748.408	0.005	12792		1
y8-NH2	771.425	771.417	-0.008	2290		1
y8	788.462	788.459	0.007	1403		1
a8	817.451	817.481	0.020	963		1
b8-H2O	827.446	827.456	0.010	1931		1
b8	845.456	845.477	0.021	1599		1
y9	916.510	916.512	0.002	1247		1
y10	1017.558	1017.560	0.002	1444		1
y11	1180.621	1180.619	-0.003	1590		1

Sample: setup_046, Component: Trastuzumab_Fc Glycosylation GOF N [61]

Fragment ion	Fragment ion mass (Da)	Peak mass (Da)	Mass error (mDa)	Intensity (Counts)	Modifiers	Charge
b13	1410.779	1410.773	-0.005	2947		2
y13	1431.723	1431.689	-0.035	1256		2
b15	1624.910	1624.909	-0.001	4213		2
y10	1752.903	1752.905	0.002	9013		2
b17	1868.905	1868.979	0.075	4019		2
y10	2110.940	2110.940	0.000	13423		2
b20	2213.216	2213.211	-0.005	11198		2
y21	2316.146	2316.127	-0.019	6181		3
a22	2411.316	2411.327	0.011	7856		3
b22	2419.311	2419.314	0.003	11213		3
y23	2526.187	2526.203	0.015	15376		3
b23	2518.379	2518.382	0.002	70937		3
b24-H2O	2621.417	2621.415	-0.002	68144		4
b24	2619.427	2619.425	-0.001	29524		4
b25-H2O	2724.426	2724.429	0.003	86575		4
b25	2742.436	2742.432	-0.004	27434		3

Figure 8. Example of trastuzumab Fc/2 CID-IMS top-down analysis report.

Conclusion

The described automated workflows for intact mass and top-down protein analysis facilitated the confirmation of protein terminal sequences and localization of modifications suggested by the intact mass results. This data processing workflow supported MS-MS data acquired with either CID or ETD fragmentation, overcoming the challenges of manually interpreting and reporting complex protein top-down results. The SYNAPT G2-Si HDMS System provided the unique capability to separate fragment ions by gas-phase ion mobility, generating simplified spectra for inspection and reviewing. Extracting and analyzing singly charged fragment ions from the top-down experiment enabled the terminal sequence to be readily confirmed, while multiply charged fragments provided data of the internal regions of the sequences within the mAb subunit. This ability to automate processing, fragment assignment, and organization of results in a templated UNIFI report facilitates the efficient communication of top-down data within and across biopharmaceutical organizations.

References

1. UNIFI Scientific Information System. Waters Brochure. 2015. 720004686EN.
2. Prosight PTM2.0 (<https://prosightptm2.northwestern.edu/>).

Featured Products

- [ACQUITY UPLC H-Class PLUS Bio System <https://www.waters.com/10166246>](https://www.waters.com/10166246)
- [SYNAPT G2-Si High Definition Mass Spectrometry <https://www.waters.com/134740622>](https://www.waters.com/134740622)
- [ACQUITY UPLC Tunable UV Detector <https://www.waters.com/514228>](https://www.waters.com/514228)
- [MassLynx MS Software <https://www.waters.com/513662>](https://www.waters.com/513662)
- [UNIFI Scientific Information System <https://www.waters.com/134801648>](https://www.waters.com/134801648)

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