

# AN INTEGRATED WORKFLOW FOR AUTOMATIC MAPPING OF DISULFIDE LINKAGES OF THERAPEUTIC PROTEINS USING HIGH-RESOLUTION LCMS, ETD FRAGMENTATION AND TARGETED INFORMATIC

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## OVERVIEW

- Disulfide bond formation is critical for establishing three dimensional folding and maintaining proper function of therapeutic proteins.
- Localization and assignment of disulfide bonds are therefore an important aspect in protein structural analysis.
- In this study, non-reduced peptide maps were acquired and processed for several therapeutic proteins using Waters Biopharmaceutical System Solution with Unifi.
- ETD technique was used to induce both disulfide bond cleavage and backbone fragmentation
- This integrated approach, combining high performance LC-MS<sup>E</sup>, ETD and targeted software, should be applicable for fast mapping and monitoring of disulfide linkages in the development of therapeutic proteins



Figure 1. Biopharmaceutical System Solution with UNIFI for disulfide bond mapping encompasses automated UPLC/MS<sup>E</sup> Xevo G2-S QToF data acquisition, data processing, reporting and report sign-off tools.

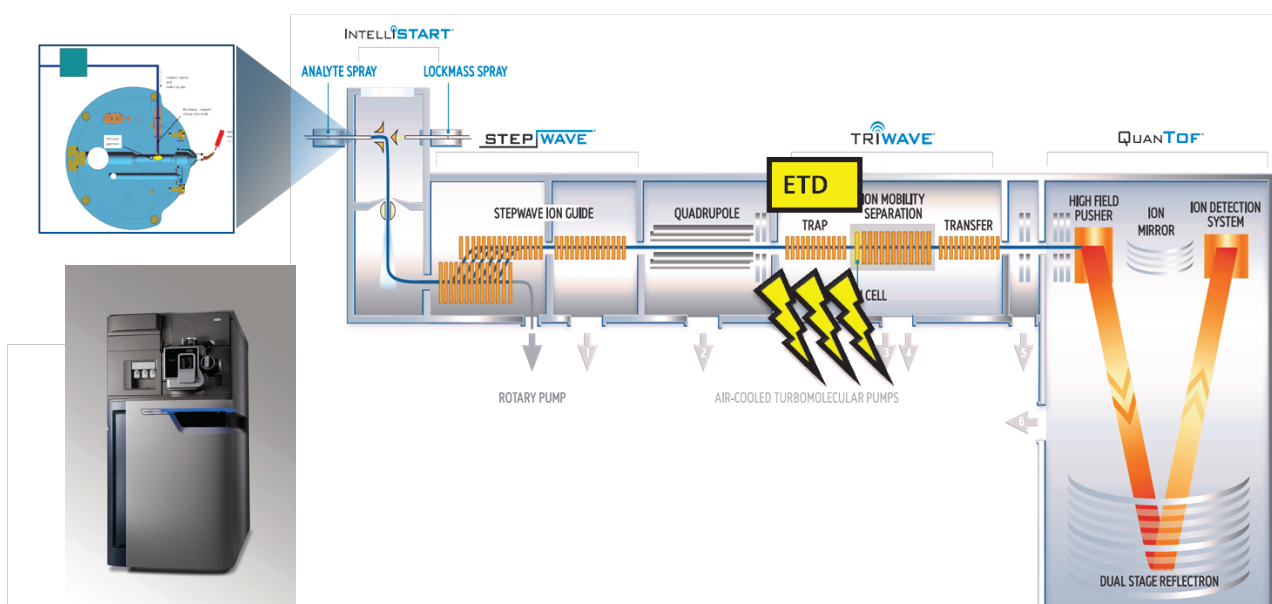


Figure 2. ETD spectra were collected using a Synapt G2-S with ETD capability

## Methods

### Waters Biopharmaceutical System Solution with UNIFI:

**LC:** Waters UPLC H-Class Bio

**Column:** ACQUITY UPLC BEH 300 C18, 2.1 x 150 mm, 1.7 μm

**Mobile Phases:**

A: 0.1% Formic Acid in Water  
B: 0.1% Formic Acid in Acetonitrile

**Column Temperature:** 65 °C

**TUV Wavelength:** 214 nm

**MS:** Waters Xevo G2-S QToF:

Data Acquisition: LC/MS<sup>E</sup>  
ESI + mode  
Capillary voltage: 3.0 kV  
Sample cone: 25 V  
Source temperature: 100 °C  
Desolvation temperature: 350 °C  
Scan rate: 0.5 Sec, Mass Range: 100– 2000 m/z

Gradient Table

Time	Flow Rate	Composit Ion A	Composit Ion B	Composit Ion C	Composit Ion D	Curve
0.00	0.200	89.0	1.0	10.0	0.0	Initial
60.00	0.200	48.0	42.0	10.0	0.0	6
61.00	0.200	10.0	80.0	10.0	0.0	6
64.00	0.200	10.0	80.0	10.0	0.0	6
65.00	0.200	89.0	1.0	10.0	0.0	6
85.00	0.200	89.0	1.0	10.0	0.0	6

### Advanced Characterization of Disulfide bonds: Targeted ETD Analysis

**LC:** Waters ACQUITY UPLC I-Class

**Column:** ACQUITY UPLC BEH 300 C18, 2.1 x 150 mm, 1.7 μm

A: 0.1% Formic Acid in Water  
B: 0.1% Formic Acid in Acetonitrile

**Column Temperature:** 65 °C

**MS:** Waters Synapt™ G2-S HDMS :

Data Acquisition: Targeted analysis  
ESI + mode  
Capillary voltage: 3.0 kV  
Sample cone: 10-25 V  
Source temperature: 100 °C  
Desolvation temperature: 200 °C

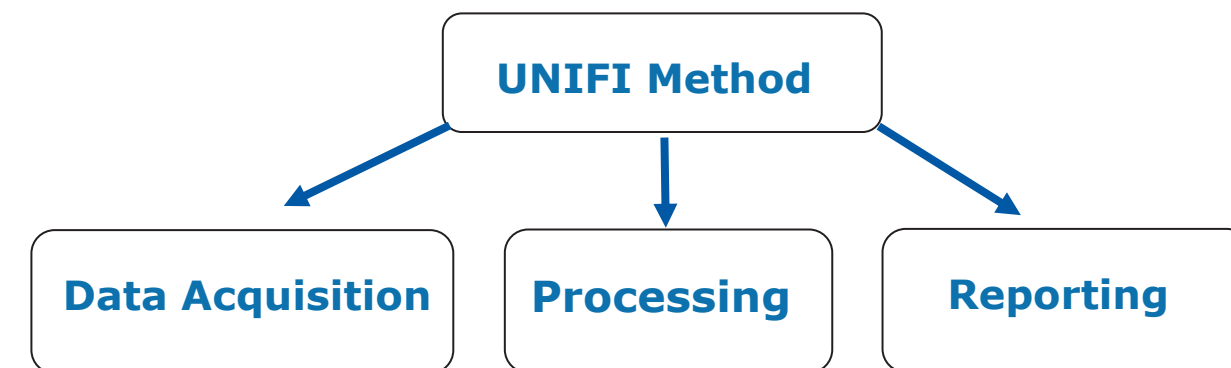
**ETD settings:**

Substance P (# 700005666)  
Reagent: 1,3-dicyanobenzene (# 700005669)  
Glow discharge current: 90 mA  
Trap wave height: 0.2-0.25 V  
Trap RF: 450-500 V

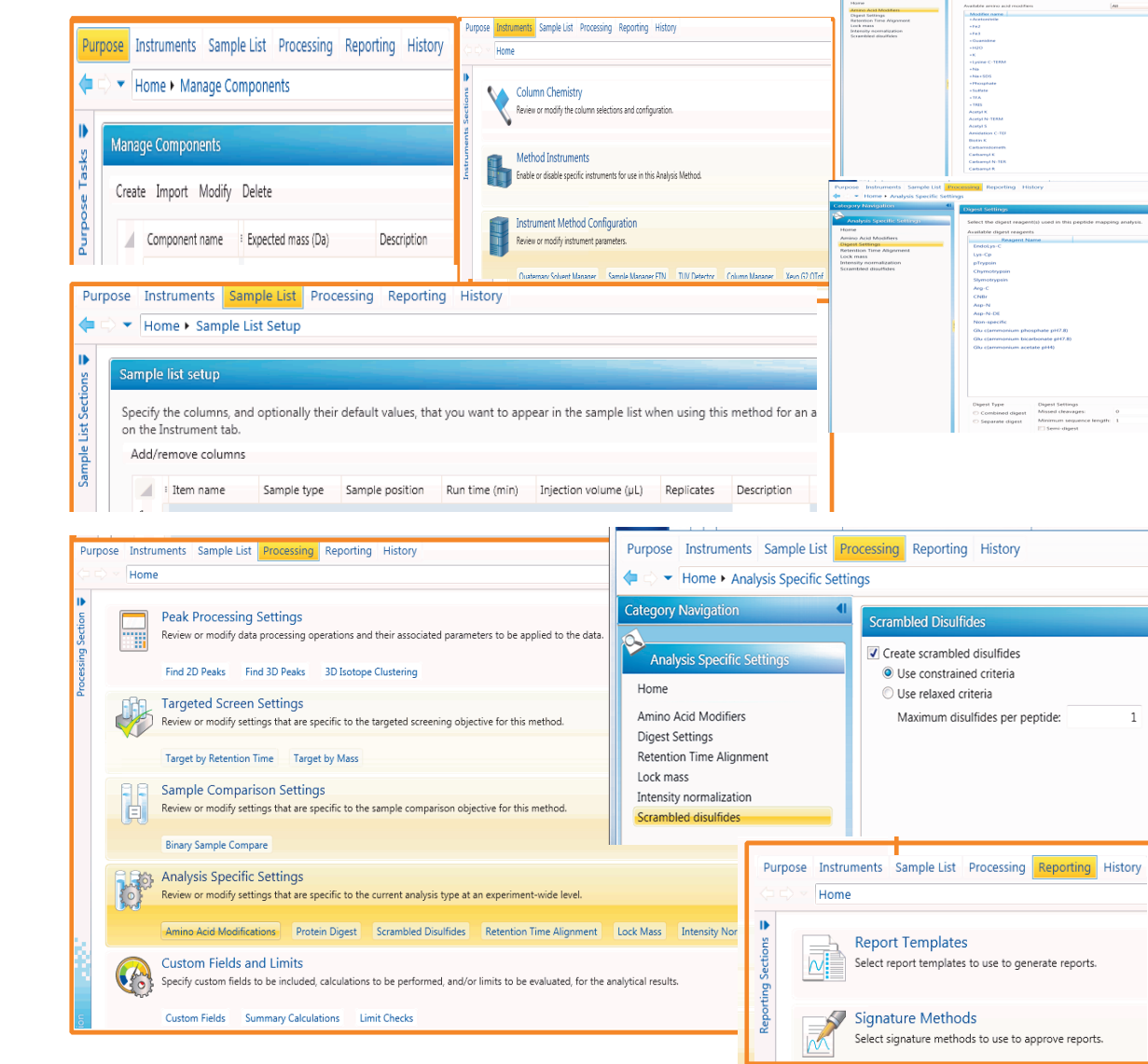
**Sample Preparation:**



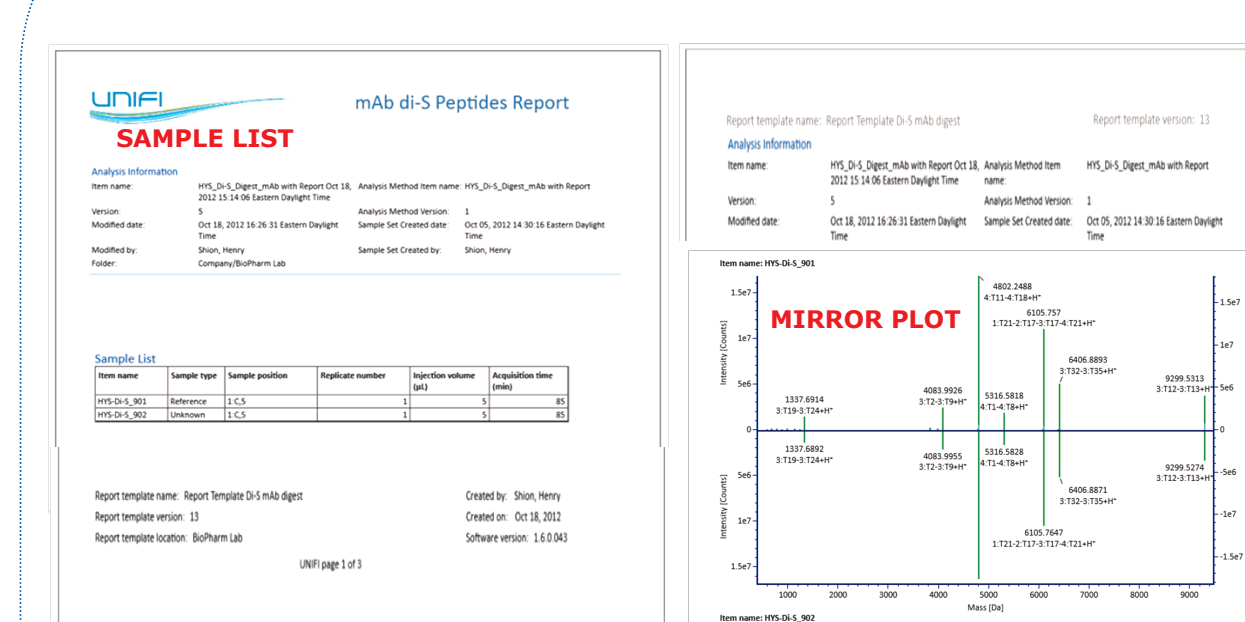
## Disulfide Linkages Analysis of mAb



### LC/MS<sup>E</sup> PEPTIDE MAPPING WORKFLOW SETUP IN UNIFI

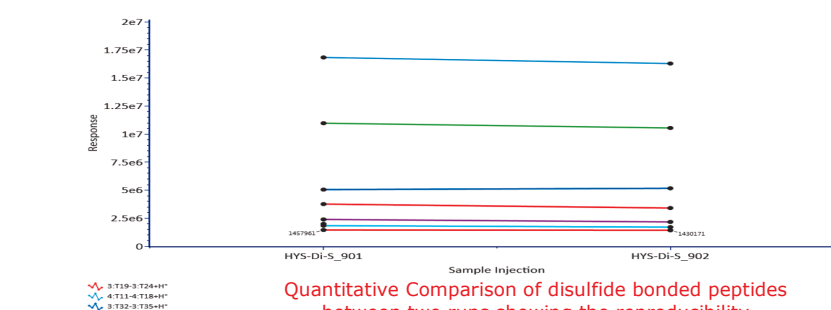


## UNIFI Reporting on mAb S-S Bond Analysis



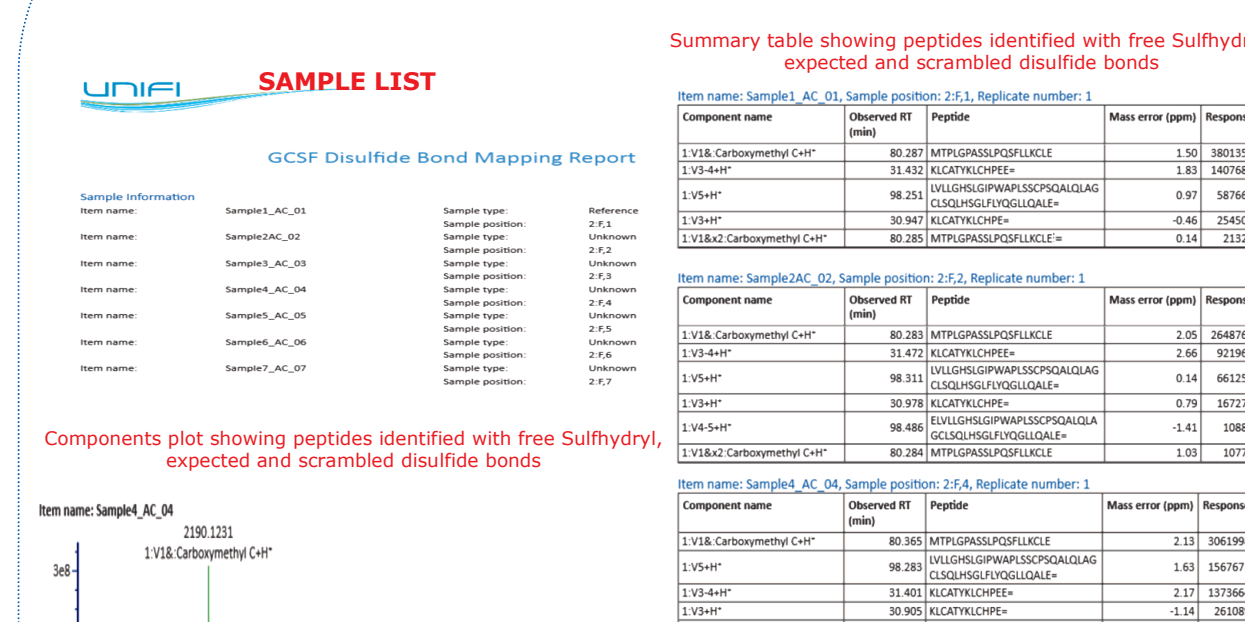
### Summary table showing peptides identified with disulfide bonds

Fragment label	Observed RT (min)	Observed mass (Da)	Expected mass (Da)	Mass error (ppm)	Peptide	Response
3-T19-3-T24	18.51	1337.6892	1337.6916	1.79	VTCVVVDSK-CR	1430171
4-T11-4-T18	38.40	4802.2478	4802.2501	0.49	ADAPVYSPFPPSSQLTSGASVGVCFNRYFP-K	16290738
3-T12-3-T35	39.19	6406.8871	6406.8968	1.51	VSLTMTDFFFDFTVEQWNGQPMENY-S	5123778
1-T21-2-T17-3-T17-4-T21	39.58	6105.7647	6105.7575	1.18	NECAGCGKPKFVYVDFVPPKPK-KGCG	10548020
3-T2-3-T9	39.81	4083.9955	4083.9943	0.30	ESGGLVAPSGSLFCTVYSGLLGVVWVR-K	2176059
4-T1-4-T8	42.12	5316.5838	5316.5902	1.38	DVLMTQTPSLPGLDGAASRCR-VEAEGLVYICQSSHPVLTGAGT	1712301
3-T12-3-T13	45.13	9299.5083	9299.5378	3.17	EPVTVWNGSLGSGVHFFRVALGDLTSSS	1204615
3-T12-3-T13	45.40	9299.5274	9299.5378	1.12	EPVTVWNGSLGSGVHFFRVALGDLTSSS	3414366



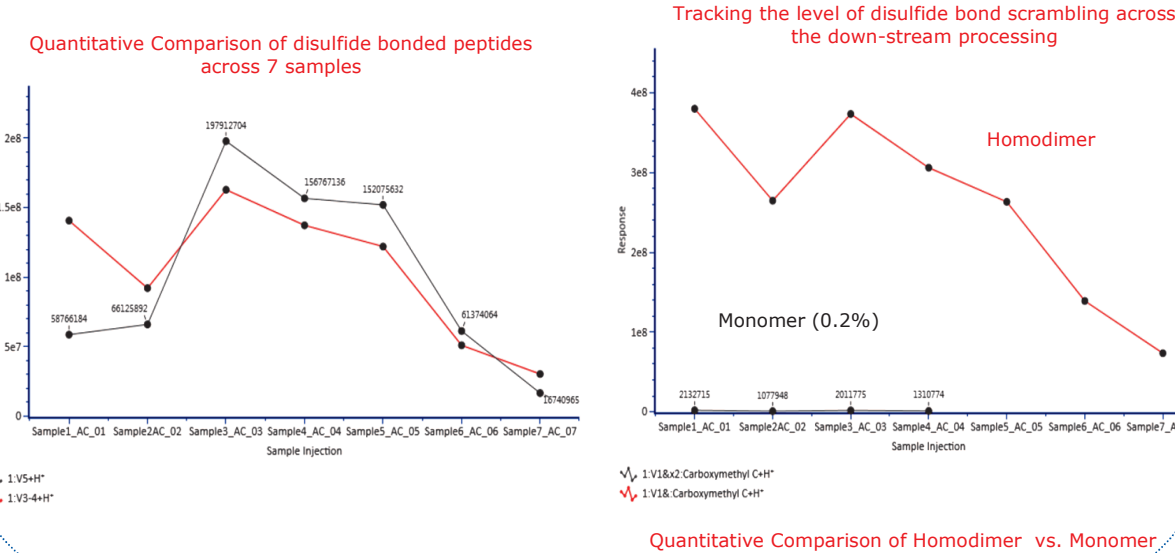
Quantitative Comparison of disulfide bonded peptides between two runs showing the reproducibility

## UNIFI Reporting on GCSF S-S Bond Analysis



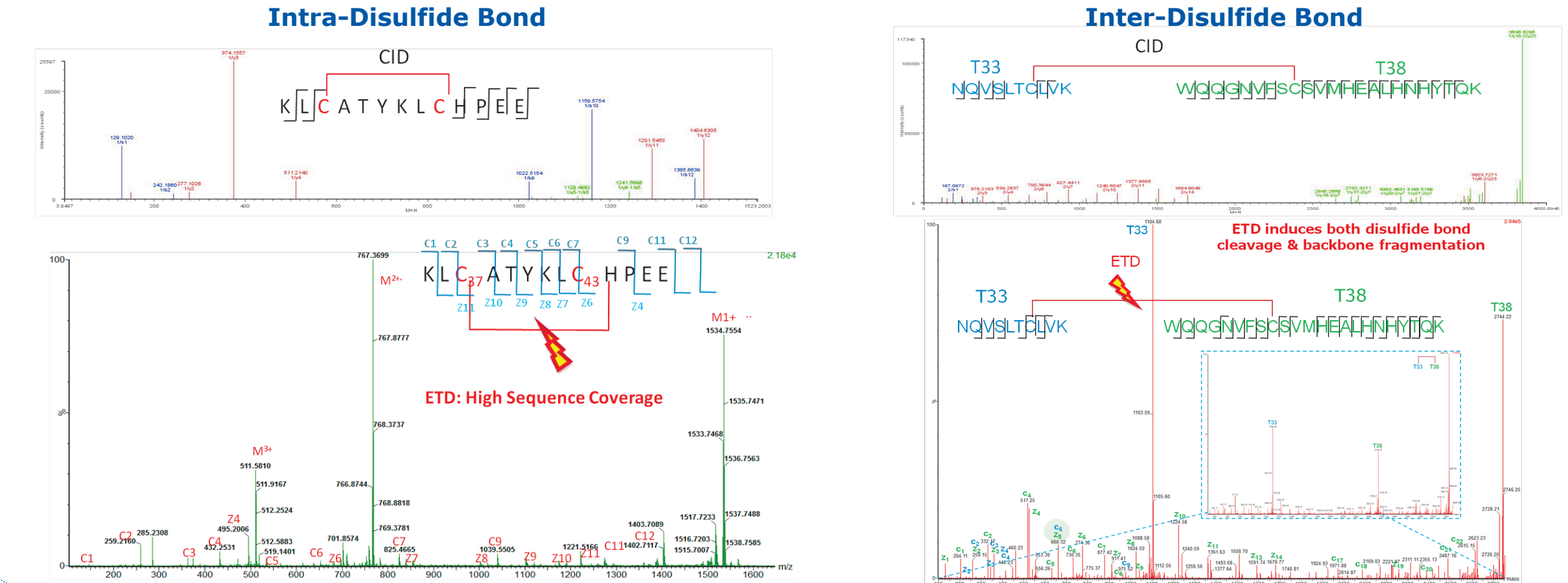
### Summary table showing peptides identified with free Sulfhydryl, expected and scrambled disulfide bonds

Component name	Observed RT (min)	Peptide	Mass error (ppm)	Response
1-V18A Carbonylmethyl Cyt	80.282	MTPLGASLPPQFLKLE	1.50	38033522
1-V18A-H	31.432	KLCAFLCHPEE	0.83	14078828
1-V18A-H	98.251	TEVLSGSPFWPSPKSPQAGASG	0.97	58766184
1-V18A-H	31.432	KLCAFLCHPEE	0.86	26002232
1-V18A2 Carbonylmethyl Cyt	80.281	MTPLGASLPPQFLKLE	0.14	2131713



Quantitative Comparison of Homodimer vs. Monomer of peptide V1

## Advanced Characterization: ETD Spectra of Disulfide Bonded Peptides



## CONCLUSIONS

- The automated mapping of disulfide linked peptides (expected or scrambled) of GCSF and mAb has been achieved using Waters Biopharmaceutical system solution with UNIFI.
- The identity of disulfide bonded peptides is automatically assigned, based on accurate MS measurement and confirmed by high-energy MS<sup>E</sup> fragment data.
- The UNIFI application workflow enables scientists in regulated or unregulated laboratory environments to acquire, process and report quantitative information about disulfide linkages in biotherapeutics, with high confidence and minimal user intervention.
- ETD fragmentation cleaved disulfide bonds, allowing sequence information from both interchain and intrachain disulfide loop regions