IMPROVING IDENTIFICATION OF SEQUENCE VARIANTS BY AN INTEGRATED MASS SPECTROMETRIC AND INFORMATICS WORKFLOW

Asish Chakraborty, Scott Geromanos, Steve Ciavarini, Matt Lauber, Weibin Chen
Waters Corporation, Milford, MA 01757, USA

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INTRODUCTION

- **Sequence variants (SV) are unintended amino acid substitution in the primary structure, and are classified as product-related impurities. The presence of sequence variants may pose concerns regarding bioactivity, stability, and immunogenicity.**
- **Sequence variants are usually present at very low-level in a therapeutic protein. From an analytical stand point, the detection and characterizing SV in a complex digest mixture that is several orders of magnitude more concentrated, remains a significant challenge.**
- **A comparative analytical strategy is presented to identify sequence variant among multiple samples. The strategy was developed and tested by analyzing monoclonal antibody samples which contain spiked synthetic peptides with amino acid substitutions.**
- **An alternative strategy is being developed to identify SV peptides in a single sample by comparing the simulated and experimental data for the primary and SV peptides based on the intrinsic physicochemical properties of peptides.**

EXPERIMENTAL

Sample Preparation:
Trastuzumab: Three tryptic digests of Trastuzumab (Digest A, B or C) were prepared separately, each at a final concentration of 2.4 picomole/µL. In digest C, two synthetic peptides of T14 containing substituted amino acid residues (see Table 2) were spiked at 1.8 femtomole/µL.
One tryptic digest of NIST mAb was used to test the single-sample workflow.

NIST mAb reference material was acquired from NIST at a concentration of 100 mg/mL in an early pilot study. The sample was digest by trypsin after reduction and alkylation.

LC/MS Conditions:

LC: **Waters ACQUITY UPLC I-Class**
Column: Acquity UPLC PST 2.1x150mm BEH C18 300Å, 1.7 µm
MS Conditions:

Instrument	Waters Synapt™ G2-Si HDMS
Mode:	ESI positive mode
Capillary Voltage:	3.0 kV
Cone Voltage:	10 V
Source Temperature:	100 °C
Desolvation Temperature:	350 °C

Informatics:
Progenesis QI; Select3D and the Simulator *(in-house software, under development)*

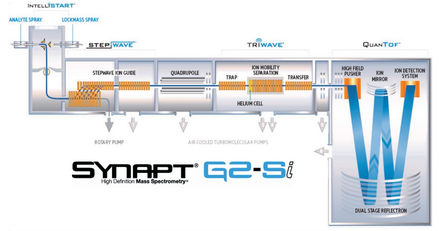

Figure 1. Data were collected using Waters *Synapt G2-Si*

COMPARATIVE WORKFLOW

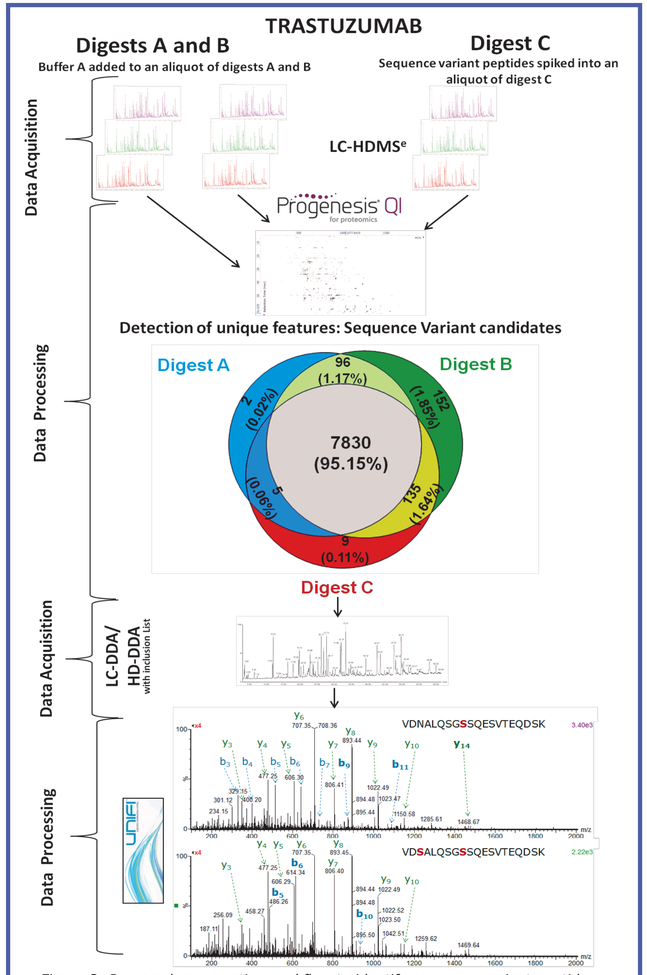

Figure 2: Proposed comparative workflow to identify sequence variant peptides.

SINGLE- SAMPLE WORKFLOW

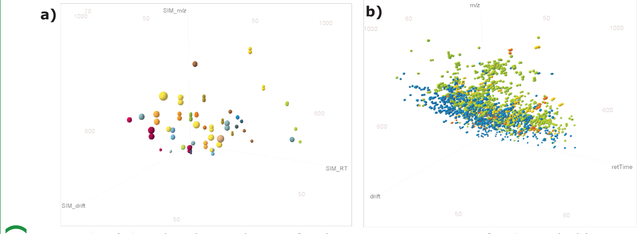

Figure 3: a) Simulated ions clusters for the primary sequence of NIST mAb. b) Experimental cluster of ions from the NIST mAb digest in a single LC-HDMS^E experiment displayed in a 3D plot based on m/z, retention time (t_r, min) and drift (t_d, Drift bins).

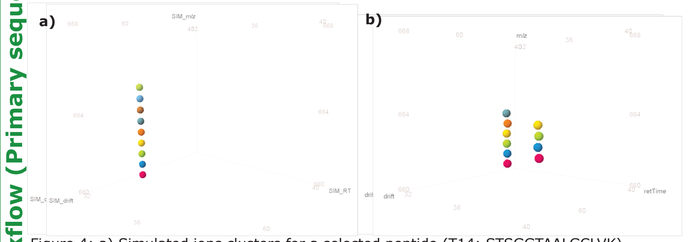

Figure 4: a) Simulated ions clusters for a selected peptide (T14: STSGGTAALGCLVK).
b) Experimental cluster of ions recorded in the same spatial volume as the simulated ions.

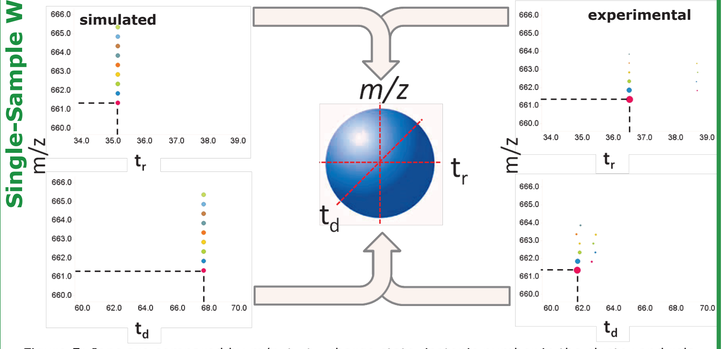

Figure 5: Ions are compared by m/z, t_r, t_d, charge state, isotopic number in the cluster and relative intensity of each isotopic peak. For the sequence STSGGTAALGCLVK only one cluster could be matched at +/- 20 ppm, 15 min and 15 drift bins.

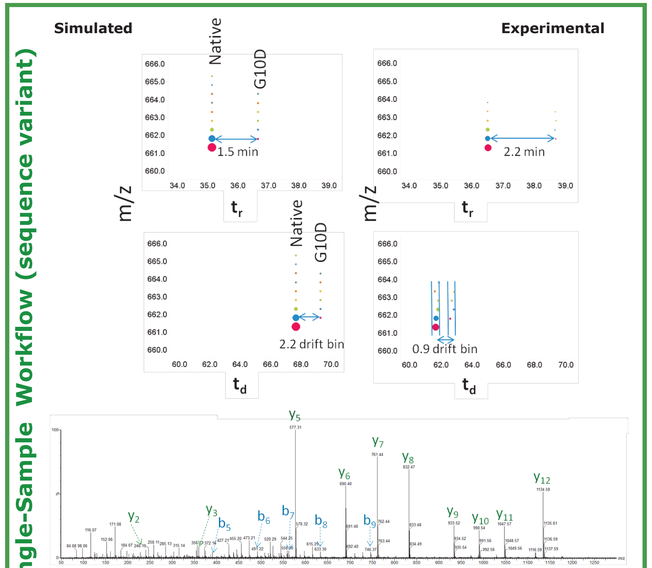

Figure 6: Sequence variant peptides were simulated, and a SV peptide of T14 (G10D, STSGGTAAL**G**CLVK) was identified based on the filtering criteria for m/z, t_r and td. An HD-DDA spectrum was subsequently acquired (lower pane) in a LC-MS experiment to further validate the substitution site.

CONCLUSIONS

Comparative workflow:

- Progenesis QI can detect and quantify low abundance sequence variant peptides (0.1%) in a monoclonal antibody digest.
- HD-DDA spectra of sequence variant peptides loaded on a 2.1 mm column at 7 femtomoles provides enough information to pin-point the substitution.

Single-Sample workflow:

- Primary sequence peptides were simulated (for each isotopic peak m/z, t_r, t_d as well as the intensity) and successfully matched to the experimental clusters of ions.
- The same approach was used to identify sequence variant peptides. A SV peptide of T14 (STSGGTAAL**D**CLVK, G10D) was used as an example to illustrate the workflow.
- High quality MS/MS spectrum was acquired using a HD-DDA method for the T14 SV peptide with fifteen b/y ions identified. The data confirms that the cluster of ions matched is SV G10D of T14 peptide.

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