A NOVEL APPROACH FOR GENERATION OF RETENTION TIME - MOLECULAR MASS CHROMATOGRAMS FOR IMPROVING INTERPRETATION AND QUANTIFICATION OF BIOTHERAPEUTIC MOLECULES.

CONNECTING SCIENCE
To Solve Problems That Matter

Waters™

Authors: Martin Green[1], Keith Richardson[1], Mark Wrona[2], Yun Alelyunas[2] Steve Bajic[1]
Affiliations: 1. Waters Corporation, Wilmslow, UK. 2. Waters Corporation Milford MA. USA

OVERVIEW

PURPOSE:

- Reduction in the complexity of chromatograms and spectra of complex Biomolecules.
- Generation and post-processing of molecular mass chromatograms.

METHOD:

- Savitzky-Golay smoothing in the chromatographic dimension.
- Deconvolution of m/z spectra over multiple RT ranges.
- Post-processing of molecular mass chromatograms.

RESULTS:

- Dramatic simplification of LC-MS Data.
- Simplified relative and absolute quantification.

INTRODUCTION

The analysis of complex mixtures of high molecular weight biotherapeutic molecules presents many challenges in mass spectrometry. The total signal is commonly split over several charge states and charge states for different species often overlap making interpretation difficult. Samples may contain multiple variants of the same component with similar UPLC retention times (RT). These challenges can lead to difficulties in identification and quantification.

In this paper we present a novel approach to transform the observed m/z spectra into chromatograms of molecular mass spectra[1], allowing direct review and post processing of deconvoluted chromatographic data. This process simplifies interpretation and quantification.

METHODS

Data were acquired using a Xevo™ G2-XS Q-ToF instrument (Waters Corporation).

ACQUITY™ UPLC ™ (Waters Corporation) H-Class PLUS Bio conditions:

Column:	ACQUITY UPLC Protein BEH C_4 300Å, 1.7µm, 2.1 mm x 50 mm
Mobile Phase:	A. Water + 0.1 % FA
	B. Acetonitrile + 0.1 % Formic Acid
Column temp:	80°C
Flow rate:	0.2 mL/min

A dilution series of Ado-Trastuzumab Emtansine (T-DM1) in serum matrix was prepared. Streptavidin-coated beads (Dynabeads M-280 Streptavidin, Thermo Fisher Scientific) were incubated with biotinylated human HER2 for two hours. After washing, serum samples (5 µL in buffer) were added and the mixture was incubated overnight at 4 °C. Post capture, beads were washed and elution of T-DM1 was performed by adding 100 µL of 10% acetonitrile in water with 1% formic acid.

RESULTS

Trastuzumab emtansine is an antibody-drug conjugate (ADC) consisting of the humanized monoclonal antibody trastuzumab covalently linked to the cytotoxic agent DM1.

Figure 1 shows the Total Ion Chromatogram (TIC) for 100 µg/mL (5 µL injection) showing significant background contamination even after purification. The inset spectrum shows the raw m/z data for the ADC at the RT indicated Illustrating the complexity of the spectrum.

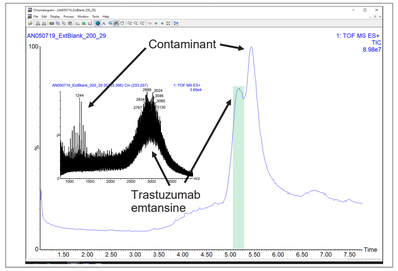

Figure 1. *m/z chromatogram and spectrum of 100 µg/mL (5 µL injection) of Trastuzumab emtansine.*

CONVERSION TO MOLECULAR MASS

LC- Mass Spectrometry (LC-MS) data comprises a three-dimensional array of retention time, mass to charge ratio and intensity (RT, m/z, I). **Figure 2** shows a plot of this array for the data in figure 1.

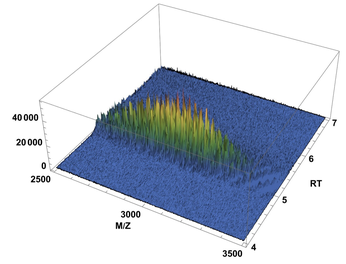

Figure 2*. Three-dimensional (RT, m/z, I) array before processing. RT range = 4 to 7 min, m/z range = 2500-3500.*

In a pre-processing step, a Savitzky-Golay smooth is applied to the data in the RT dimension **(Figure 3).** This enhances the signal-to-noise of individual m/z spectra while maintaining chromatographic peak shape and width.

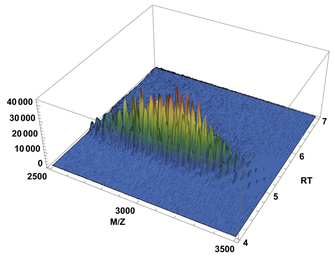

Figure 3. *Three-dimensional (RT, m/z, I) after processing with Savitzky -Golay smooth in the chromatographic dimension.*

Each pre-processed m/z spectrum is then transformed from the m/z to molecular mass (M) domain using a Maximum Entropy deconvolution algorithm[2]. This results in the generation of a new data file comprising a three-dimensional array of RT, mass, and intensity (RT, M, I) **(Figure 4)**. Signal previously split over several charge states now appears as a single peak improving S/N for quantification and decreasing spectral complexity.

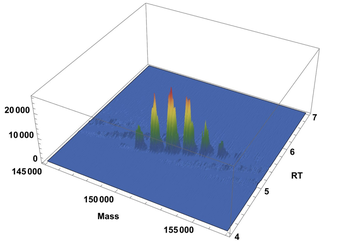

Figure 4. *Three-dimensional (RT, M, I) after Maximum Entropy deconvolution of smoothed data.*

A simple user interface **(Figure 5)** was developed to read in chromatographic data and generate a transformed data file (RT, M, I). Maximum Entropy parameters used were, output range 140kDa to 160kDa, m/z input range 2500-3500 and retention time range of 4 to 7 min. This process results in dramatic simplification of the chromatogram **(Figure 6).**

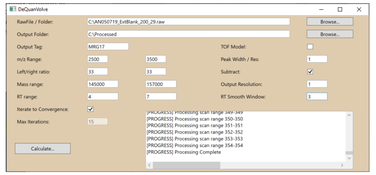

Figure 5. *Research prototype user interface.*

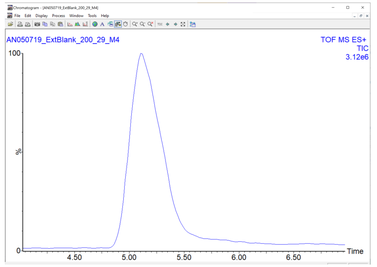

Figure 6 *Molecular mass, Total Ion Chromatogram after processing. Each data point comprises a molecular mass spectrum. The chromatogram may reviewed and processed as normal.*

Figure 7 shows molecular mass spectrum summed over the chromatographic peak in Figure 6. The spectrum is easily interpretable, clearly showing the glycoform and drug antibody conjugation information.

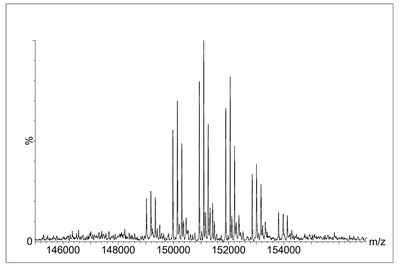

Figure 7 *Molecular mass spectrum from data summed over chromatographic peak in Figure 6.*

Figure 8 shows the reconstructed molecular mass chromatograms for individual Glycans/ Drug-to-antibody ratio's (DAR's) showing chromatographic retention time range for individual components, simplifying quantification.

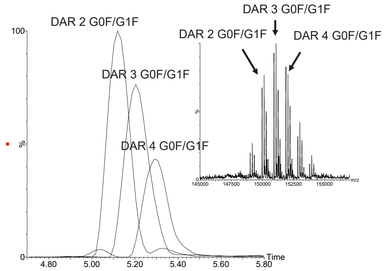

Figure 8 *Molecular mass Chromatographic profiles for a specific glyco-form with varying drug-antibody conjugation.*

Figures 9 and 10 show quantification results from a serial dilution of the sample for two individual Glycoforms of DAR3 over a concentration range 0.5 µg/mL to 100 µg/mL processed directly from the molecular mass chromatogram.

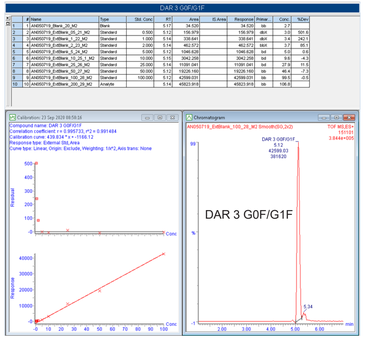

Figure 9. *Linear quantification results for DAR 3 G0F/G1F series dilution 0.5µg/mL to 100µg/mL (5µL injection).*

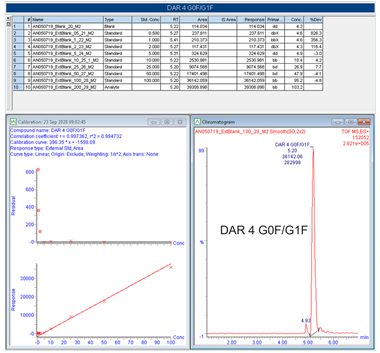

Figure 10 *Linear quantification results for DAR 4 G0F/G1F series dilution 0.5µg/mL to 100µg/mL(5 µL injection).*

DISCUSSION

This analysis of an antibody drug conjugate demonstrates the complexity of the data generated, even after purification.

Transposition from an m/z chromatogram to a molecular mass chromatogram dramatically simplifies the data, separating overlapping charge envelopes and revealing details of chromatographic elution profiles, allowing quantification using standard software workflows.

The deconvolution process described, combines information from the entire charge state envelope for each species maximising statistical precision as well as improving signal-to-noise. This general approach is also applicable to many other types of data where multiple overlapping charge states increase complexity including data from other mass analysers, such as scanning quadrupole mass filters.

CONCLUSION

- **Generation of molecular mass chromatograms simplifies interpretation and quantification.**
- **Novel approach preserves chromatographic fidelity while maximising statistical precision for deconvolution**
- **Further work planned to expand method to other applications and quadrupole mass filter data.**

References

1. K.M. Millea et al., Evaluation of multidimensional (ion-exchange/reversed-phase) protein separations using linear and step gradients in the first dimension, *J. Chromatogr. A 1079 (2005) 287–298.*
2. A. Ferrige, M. Seddon, B. Green. S. Jarvis, J. Staunton. Disentangling electrospray spectra with maximum entropy. *Rapid Commun. Mass Spectrom. 6 (1992), pp. 707-711.*

TO DOWNLOAD A COPY OF THIS POSTER, VISIT WWW.WATERS.COM/POSTERS