

Summary

The size and heterogeneity of modern biotherapeutic molecules present unique challenges to analytical pipelines

Novel analytical and computational approaches are required to accurately characterize and quantify complex biologics.

Progressive deconvolution of intact mass spectrometry analyses coupled with multi dimensional feature detection robustly addresses such needs

Introduction

Erythropoietin, commonly referred to as EPO is a highly glycosylated glycoprotein with approximately 40% of its weight composed of sugars, which prompts the need to find ways to characterize important critical quality attributes like sialylation and antennary glycans. EPO has historically been difficult to characterize by traditional approaches due to the heterogeneity resulting from heavy glycosylation with varying abundance levels. It is therefore desirable to develop analytical methods using fit-for-purpose instrumentation and reproducible sample preparation to reveal its complexity and detect all species present even at low levels. Here, we highlight a sensitive feature detection data processing approach, which is of significant interest.

Methods

Two different batches of EPO from the same manufacturer were treated with specific enzymes to enable glycoprofile analysis by desialylation (SialEXO[®]), specific hydrolysis of O-glycans (OglyZOR[®]), and removal of all N-glycans using PNGase F.

All samples were analyzed by RP-LC-MS intact protein analysis using a QToF mass spectrometer. For data processing Parsimonious intact deconvolution algorithm was used in the Byos software by Protein Metrics Inc. Data was processed via Trace Peak deconvolution and with Progressive Deconvolution.

The workflow for large mass feature detection with progressive deconvolution begins with the sampling of short, fixed-width, overlapping segments of the chromatogram (a). The resulting deconvolved mass candidates from these trace segments (b) are transformed into a mass-time matrix and multi-dimensional segmentation provides elution times of unique masses (c). Subsequent peak segmentation of an extracted mass chromatogram identifies peak bounds of the eluting species and can distinguish isomeric forms if they exist. Results are organized and viewable in a user interface (d).

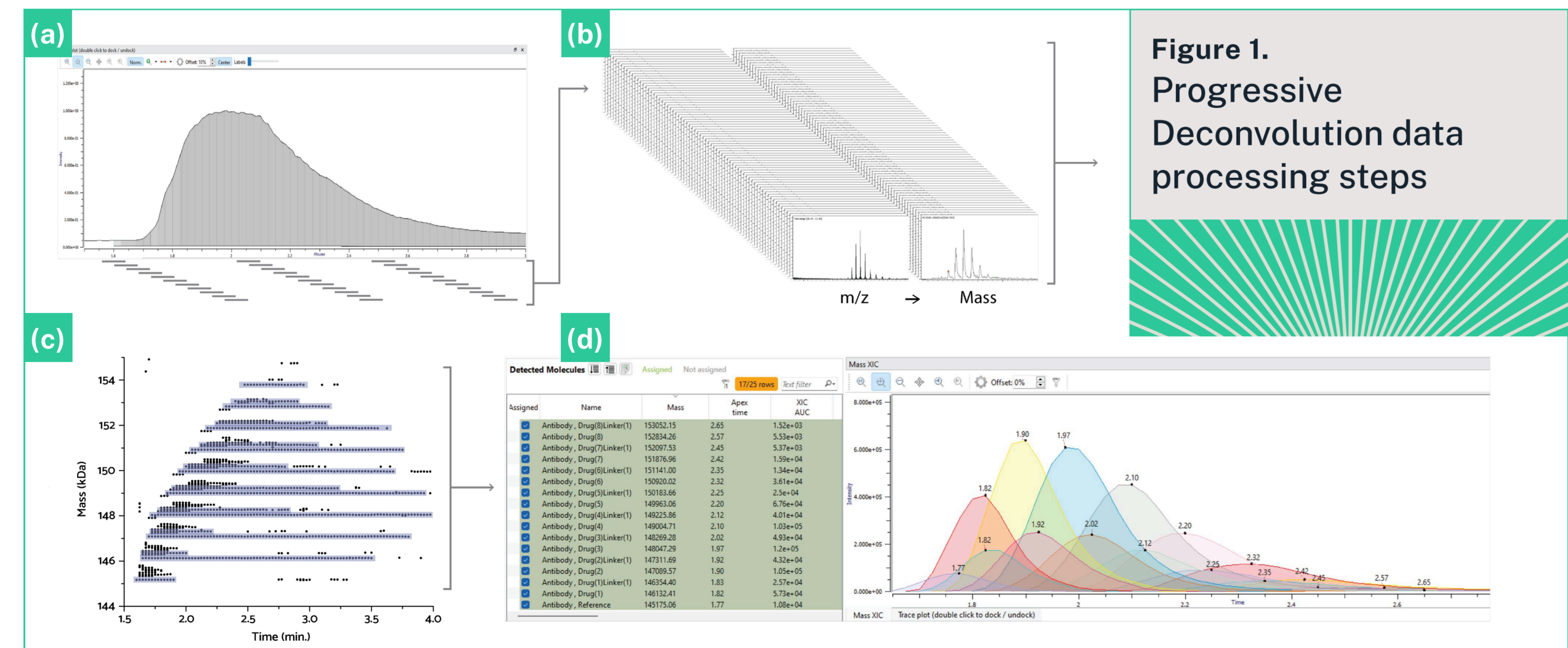


Figure 1. Progressive Deconvolution data processing steps

Results

Traditional Deconvolution results showing N-Glycans and O-Glycans after enzymatic treatment

Reducing complexity of intact protein MS spectra for routine and fast analysis of EPO is highly desirable to investigate potential modifications masked by glycosylation heterogeneity.

- EPO has
 - 3 x N-Linked glycan sites, 1 x O-Linked glycan
 - 1-2 disulphide bonds
 - Average mass (Protein only) 18237

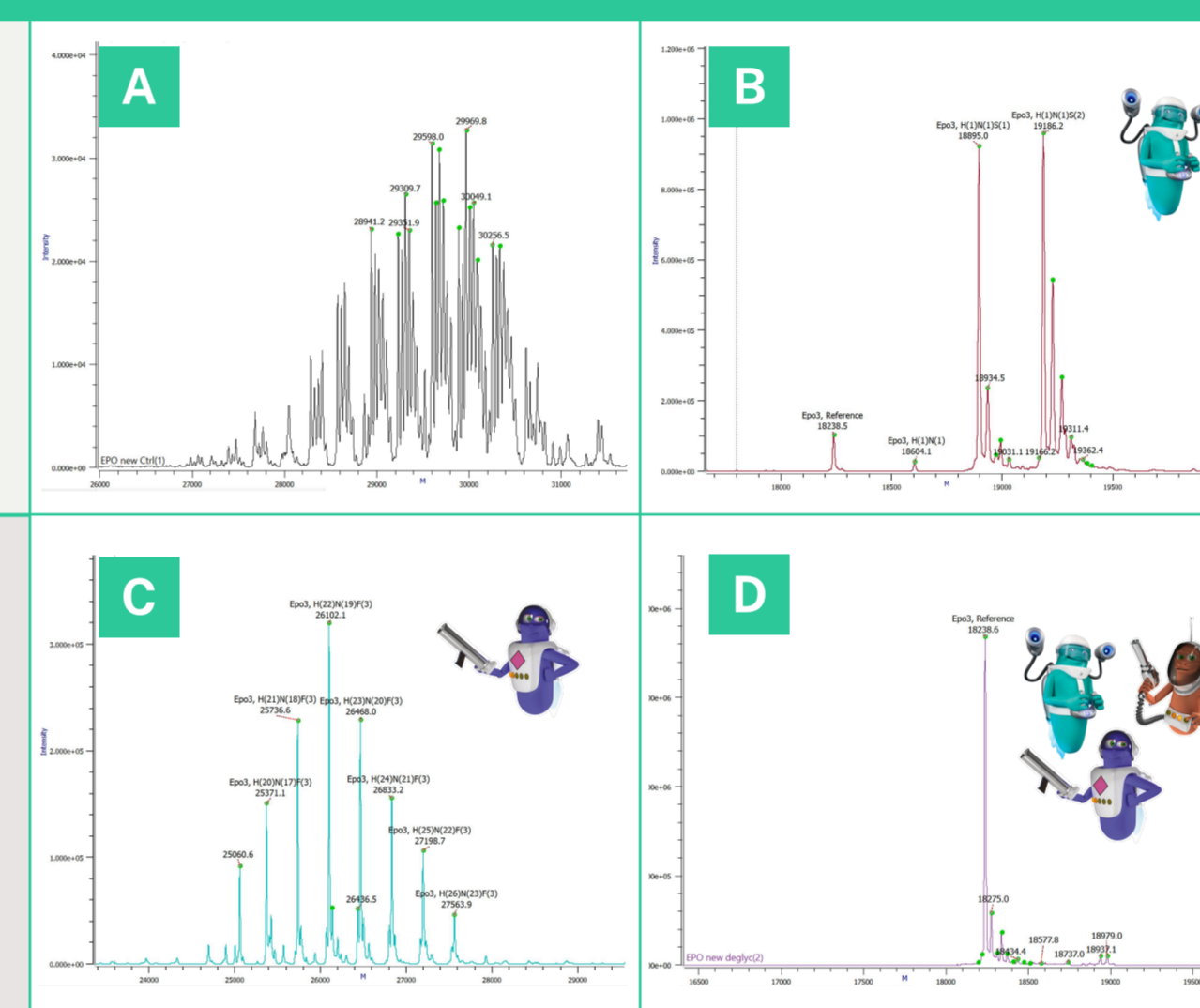


Figure 2. Deconvoluted MS Spectra for different samples

A. Control without treatment
B. PNGase F treated - distribution of O-glycans
C. SialEXO - desialylated sample
D. All enzymes combined

mass XICs showing the distribution of different glycan species and allowing alternative quantitation to relative intensity-based quantitation

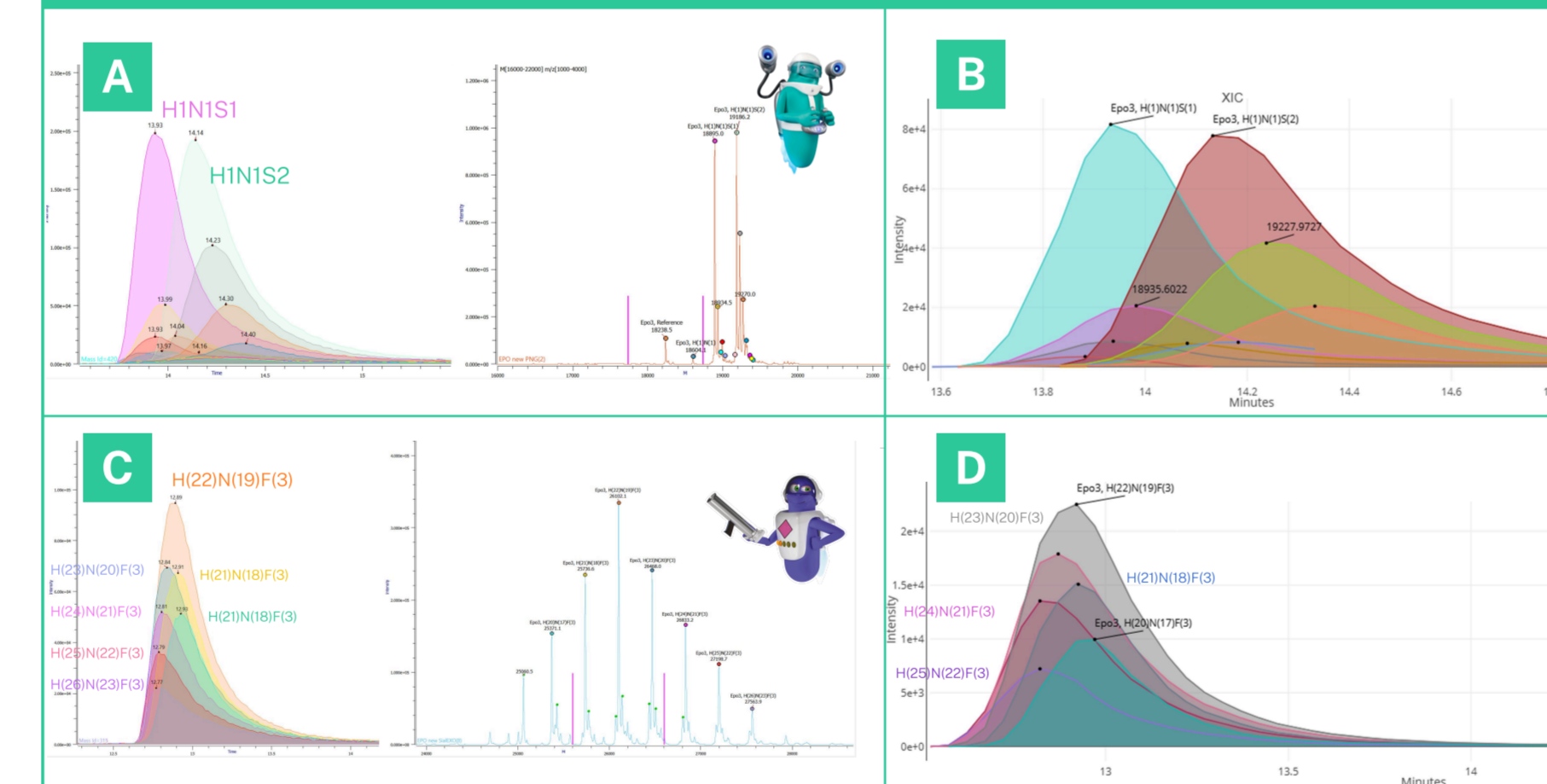


Figure 3. mass XICs from traditional and Progressive Deconvolution data

A. Trace Peak Deconvolution PNGase F treated
B. Progressive Deconvolution PNGase F
C. Trace Peak Deconvolution SialEXO
D. Progressive Deconvolution SialEXO

The distribution of the different glycoforms is very similar for the PNGase F sample, but Figure C and D highlight a few small differences for XIC apices, shapes and intensities for various Glycosylated species when Progressive Deconvolution is used.

Comparison of Quantitation based on Intensity and XIC AUC

Sample name	EPO am PNG	EPO am SialEXO	EPO id SialEXO
Peak #	2 (%)	5 (%)	9 (%)
Epo3_Reference	5.31		
Epo3_H2S6N2S2P3D	3.85	4.72	
Epo3_H2S5N2S2P3D	6.63	9.78	
Epo3_H2S4N2S2P3D	12.84	13.62	
Epo3_H2S3N2S2P3D	18.68	19.16	
Epo3_H2S2N2S2P3D	25.95	24.37	
Epo3_H2S1N2S2P3D	18.16	17.08	
Epo3_H2S0N2S2P3D	11.93	11.06	
Epo3_H2S6N1S2P3D	47.42		
Epo3_H2S5N1S2P3D	43.66		
Epo3_H2S4N1S2P3D	1.61		

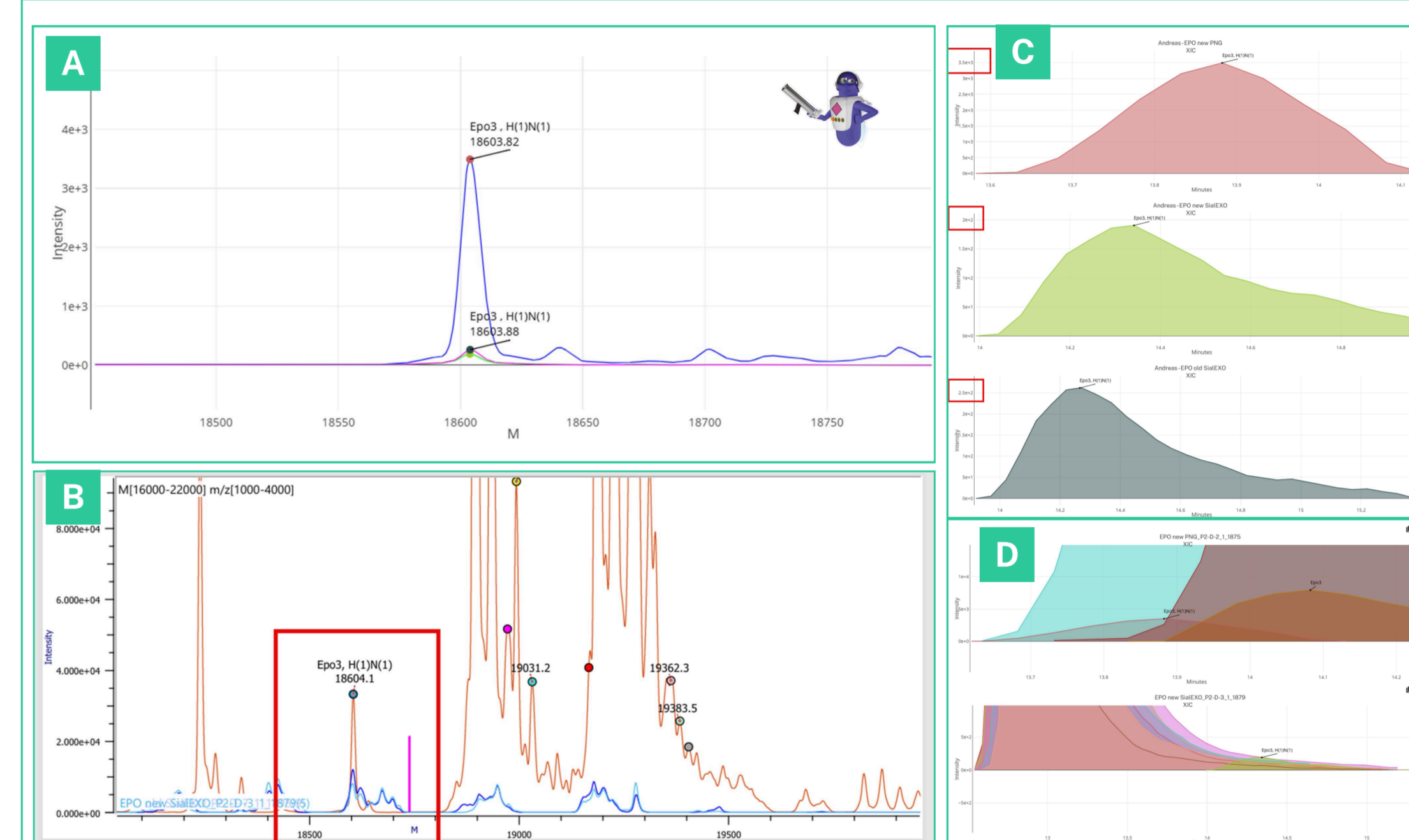
Table 1. Relative Quantitation of different species in the PNGase F and SialEXO treated samples

A. Trace Peak Deconvolution Relative quantitation using Intensity of species in the Deconvoluted spectra
B. Progressive Deconvolution Relative Quantitation using XIC AUC values for the different detected species

The reported relative abundance values are very similar, but lower-level detected species do modify the overall picture. H(1)N(1) is detected in the Progressive Deconvolution processed samples.

Results – Detection of Low abundance species

Reducing complexity of intact protein MS spectra for routine and fast analysis of EPO allows the investigation of potential modifications by glycosylation heterogeneity. With Progressive Deconvolution – due to increase of the dynamic range for the detected deconvoluted species – additional masses can be identified with high confidence.



After the massXICs based dynamic reports highlighted the presence of the H(1)N(1) in the SialEXO treated samples. Closer inspection confirmed that the mass peak is indeed present and correctly matched with high confidence – supported by the minimum consecutive slices feature for Progressive Deconvolution.

Figure 4. Detection of Low abundant species with Progressive Deconvolution in SialEXO treated samples
A. SialEXO Treated sample with Progressive Deconvolution - H(1)N(1) detected and matched by software
B. SialEXO Treated sample with Trace Peak Deconvolution
C-D. mass XIC for H(1)N(1) in different samples showing intensity differences and wider dynamic range for detection.

Conclusion

- Protein Metrics' parsimonious deconvolution algorithm is a best-in-class option for deconvolution of intact mass spectra in both trace peak deconvolution and progressive deconvolution applications
- The progressive deconvolution method presented here proved to be a robust and sensitive feature detection tool to assure lower abundance intact species of different glycoforms of EPO are detected with high confidence.
- Relative Quantitation of glycoforms present in the sample can be performed by utilizing the resulting massXICs – which are providing a more comprehensive assessment of relative levels across the time range of the progressive deconvolution experiment. Results are comparable to conventional trace peak deconvolution methods.
- Progressive Deconvolution yields additional high confidence IDs compared to conventional methods of trace peak-based deconvolution which improvement can be contributed to the higher validated dynamic range.