Glycopeptide profiling in a trapped ion mobility quadrupole time-of-flight mass spectrometer (timsTOF Pro)

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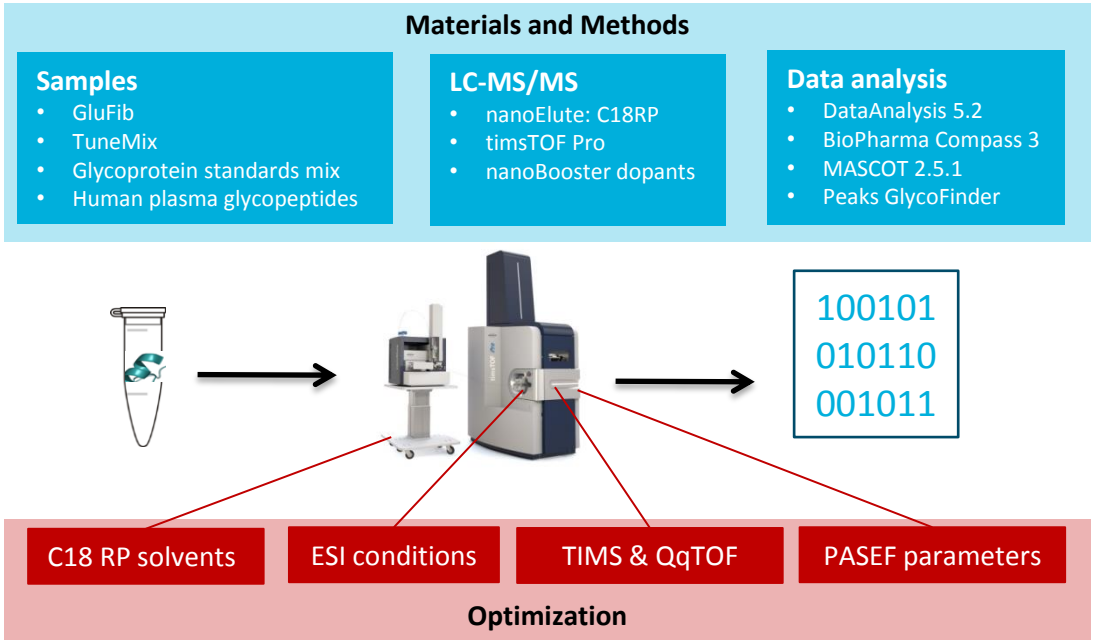
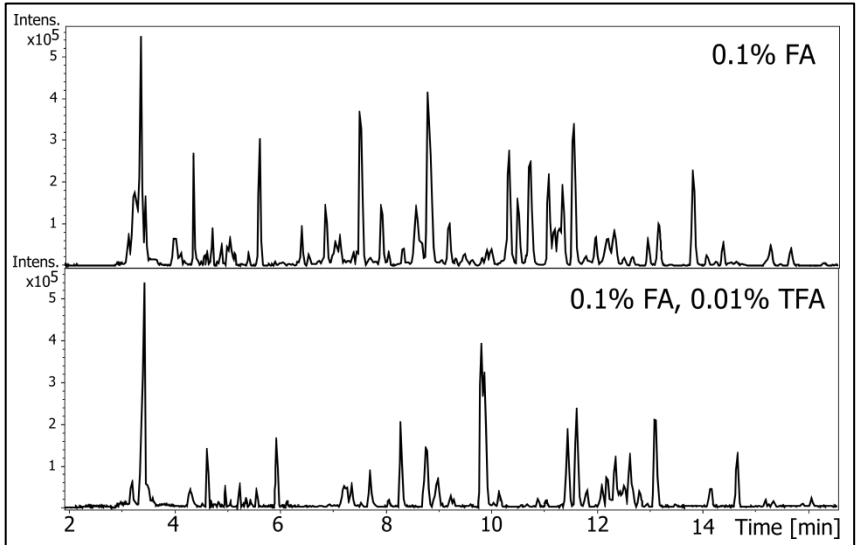
Introduction

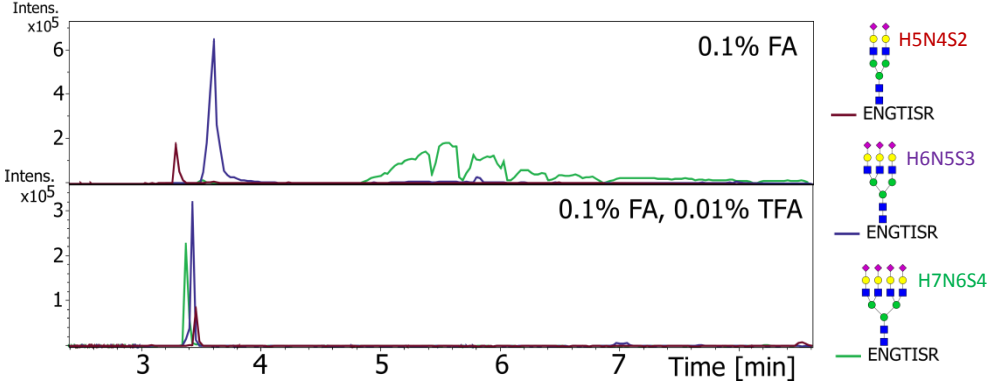


Recent introduction of parallel accumulation serial fragmentation (PASEF) on a trapped ion mobility quadrupole time-of-flight mass spectrometer provides unique possibilities for comprehensive glycopeptides profiling in complex samples such as blood plasma. Glycopeptide profiling of patient samples holds great potential to diagnose, monitor and understand both genetic and acquired human diseases. Glycosylated peptides present analytical challenges for LC-MS/MS analysis with respect to chromatographic separation, electrospray ionization, and structural elucidation by collision induced dissociation experiments. In this work we set out to optimize the data generation workflow on the timsTOF Pro platform for N-glycopeptide profiling. Parameter optimizations discussed in this communication are: C18RP mobile phase composition (**slide 3**), TIMS and QqTOF ion optics (**slide 4**), ESI conditions (**slide 5**) and PASEF parameters (**slides 6-8**).

Improved C18RP separation of glycopeptides by TFA ion pairing



Base peak chromatograms of tryptic digest of model glycoproteins (TRFE_HUMAN, A1AG1_HUMAN, FETUA_BOVIN, Trastuzumab) using 0.1% FA and 0.1% FA, 0.01% additives in H2O (solvent A) and ACN (solvent B). Gradient: linear increase of 3-45% solvent B in 15 minutes at 500nl/min. Trapping column: Acclaim C18RP RLSC (0.075 x 20mm, 3µm particles, 100Å pore size; Thermo scientific). Analytical column: C18RP nanoElute FIFTEEN analytical column (0.075 x 150mm, 1.9µm particles, 120Å pore size; Bruker Daltonics) operated at 45˚C.

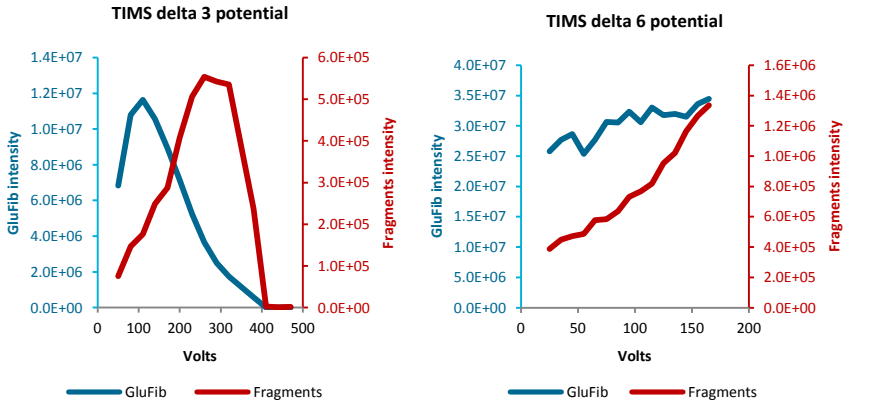


	APEX Rt (min)		Full width (sec)		Tailing	
Glycoform	FA	FA TFA	FA	FA TFA	FA	FA TFA
H5N4S2	3.3	3.46	7.3	5.8	1.403	1.086
H6N5S3	3.61	3.43	14.8	6.7	0.966	1.027
H7N6S4	5.56	3.38	165	6	N.D.	1.282

Extracted ion chromatograms of disialo-, trisialo-, and tetrasialo glycoforms of peptide ENGTISR from human Alpha-1-acid glycoprotein 1 (A1AG1_HUMAN). With 0.1% FA solvent composition glycoforms are separated according to their respective number of sialic acids. The EIC of the tetrasialo glycoform of ENGTISR shows extreme peak broadening and tailing with 0.1% FA. Addition of 0.01% TFA significantly improves chromatographic separation of all glycoforms but achieves a remarkable 27.5x reduction in full peak width for the tetrasialo glycoform.

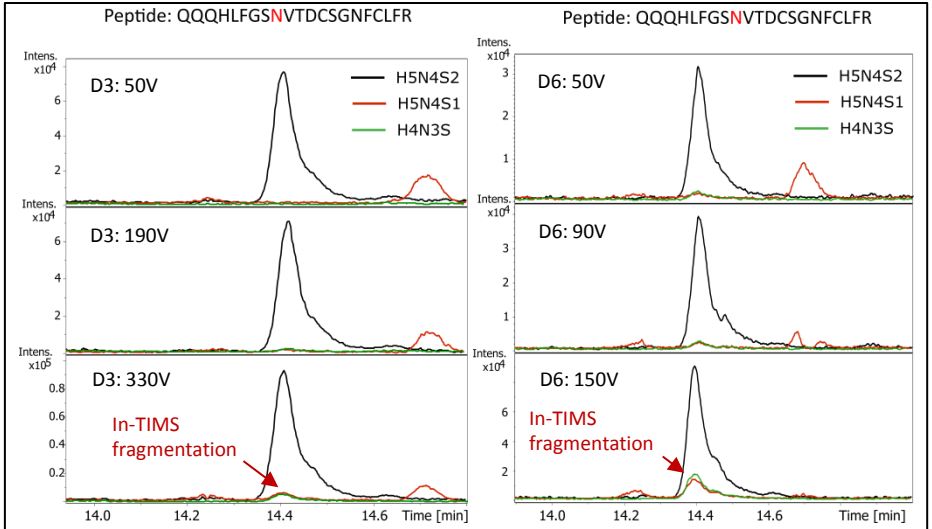
Reversed phase C18 liquid chromatography separation of glycopeptides with typical 0.1% formic acid (FA) solvent composition performs poorly for highly sialylated glycopeptides. Glycoforms of the same peptide show increased retention, peak broadening and increased tailing when the number of sialic acids in the glycan structure increases. Addition of low levels of trifluoroacetic acid (TFA) as ion pair reagent mitigates interactions between sialic acids and the stationary phase leading to near co-elution of all glycoforms of a peptide with sharp chromatographic peaks with minimal tailing. Signal intensity due to ion suppression by TFA is only ~50% thanks to acetonitrile nanoBooster dopant.

TIMS delta potentials and ion optics tuning



Dual axis intensity plots of GluFib direct infusion experiments at various delta 3 and delta 6 potentials. For Delta 3 potential ramping Delta 6 was set to default 50V. For Delta 6 potential ramping the Delta 3 potential was set to default 110V.

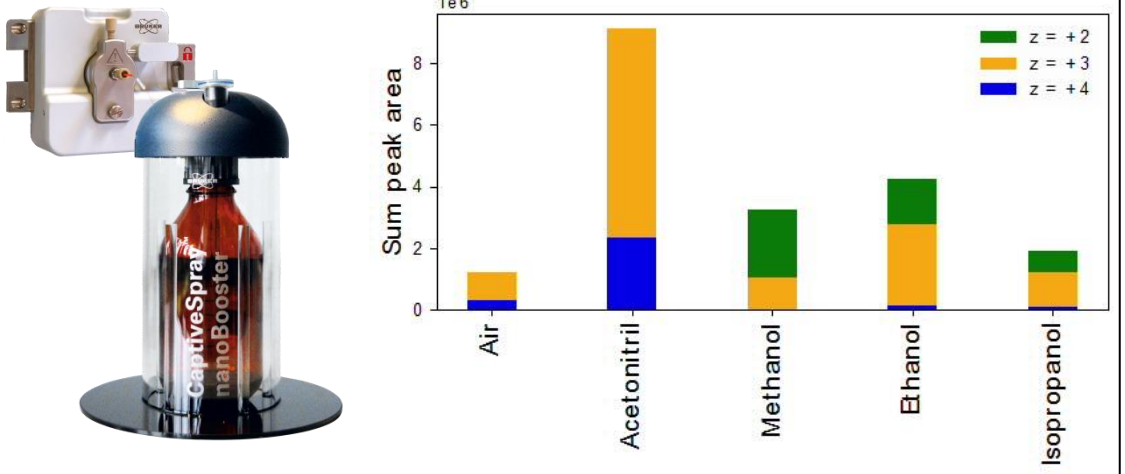
TIMS		MS1 and low CE MS2		high CE MS2	
D1	-20V	Transfer time	100µs	Transfer time	64µs
D2	-150V	Collision RF	2000Vpp	Collision RF	1600Vpp
D3	110V	Pre Pulse Storage	13µs	Pre Pulse Storage	10µs
D4	110V				
D5	0V				
D6	55V				



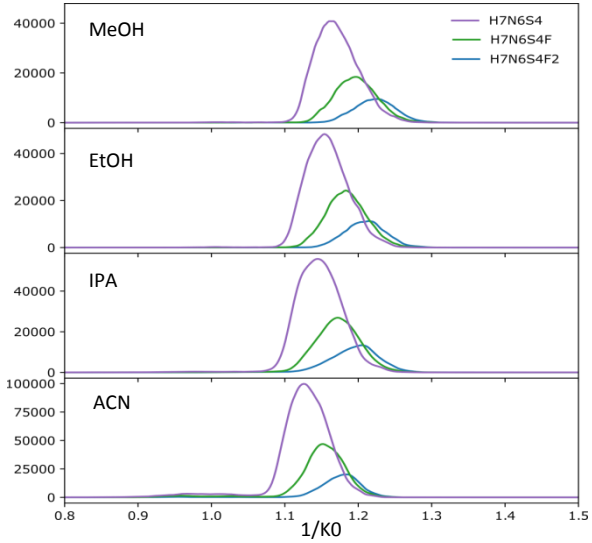
Extracted ion currents for the intact Hex5HexNAc4NeuAc2 glycoforms of the N630 tryptic glycopeptide of Transferrin (TRFE_HUMAN) and its subsequnt sialic acid and antenna losses (Hex5HexNAc4NeuAc1 and Hex4HexNAc3NeuAc1, respectively) due to in-TIMS fragmentation. Delta 3 (D3) potentials were set to 50, 190 and 330 V with Delta 6 (D6) potential at default 50V. Delta 6 (D6) potentials were set to 50, 90 and 150V with D3 at default 110V.

TIMS delta potentials and QqTOF ion optics were tuned for optimal transmission of intact glycopeptides using tunemix and GluFib direct infusion as well as LC-MS analysis of glycopeptides from glycoprotein standards. First we optimized transmission by ramping individual TIMS delta potentials whilst monitoring intact analyte ion transmission as well as detectable fragment ions. GluFib results show that delta 3 and delta 6 TIMS potentials lead to undesired in-TIMS fragmentation when set to high values. Glycopeptides measurements indicate that delta 6 TIMS potential is primarily responsible for in-TIMS fragmentation. QqTOF ion optics were optimized using tunemix for optimal mass range transmission of both intact glycopeptides in MS1 or glycan-moiety fragments in low collision energy MS2 experiments. Finally, we also optimized ion optics parameters for transmission of peptide-moiety fragments generated in high collision energy MS2 experiments. Optimal key parameters are listed in the table.

nanoBooster ESI nitrogen source gas dopants



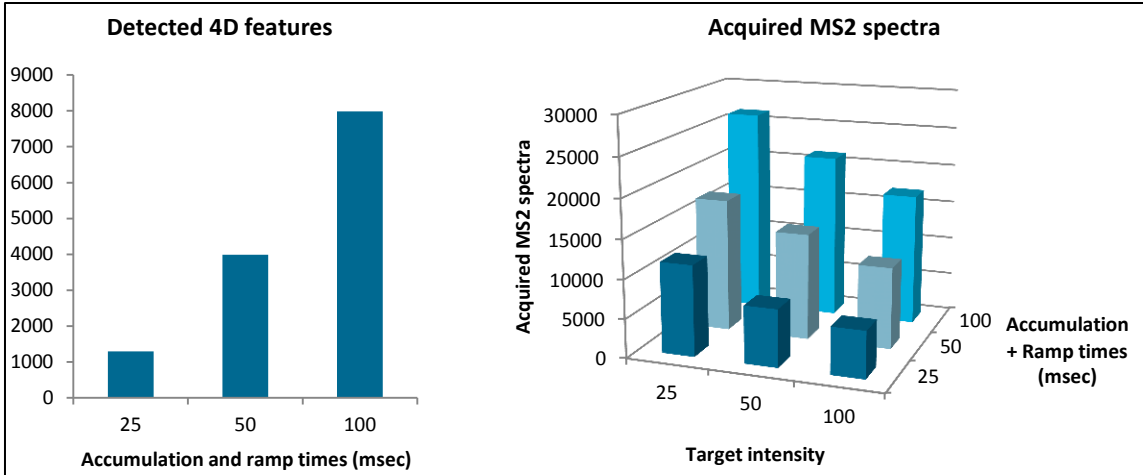
CaptiveSprayer with nanoBooster image and bar graph of summed peak areas of glycopeptides from a tryptic digest mixture of TRFE_HUMAN, A1AG1_HUMAN, FETUA_BOVIN and Trastuzumab using different nanoBooster dopants. Acetonitrile provides highest possible glycopeptide signal intensities and highest precursor ion chargestates for optimal CID fragmentation of peptide-moieties.



Extracted ion mobilograms for selected glycoforms from the tetrasialo N-glycopeptide E**N**GTISR from A1AG_HUMAN using different nanoBooster dopants.

Glycopeptides typically suffer from poor ionization efficiency in electrospray ionization. Use of dopant enriched N2 gas in the captive sprayer ESI source significantly enhances the ionization efficiency of glycopeptides but also results in supercharging (acetonitrile) or subcharging (primary alcohols) of ions depending on the solvent used. NanoBooster solvents have a significant impact on TIMS separation as the increased gas density leads to an increased collisional force that pushes ions deeper into the TIMS funnels. Positioning of ions within the TIMS funnels was corrected by reducing the gas velocity and polyalanine as collisional cross section standards was used to calibrate reduced mobility values (1/K0). In general, only minor shifts in 1/K0 values could be observed for the vast majority of glycopeptides with no notable differences in mobility peak shape, number of mobility features or resolution. However, sporadic cases could be observed where both 1/K0 as well as the mobility peak shapes of glycopeptide ions were significantly influenced in a dopant specific manner.

Optimization of PASEF parameters



Results obtained for enriched glycopeptides from control plasma at various accumulation + ramp times in combination with various target PASEF intensities. Glycopeptides were separated using 60 minute linear gradients of 3-45% acetonitrile in 0.1% FA, 0.01% TFA at 500nl/min. 4D feature detection was performed using Peaks studio X+ (BSI). MGF files were generated using DataAnalysis 5.2 and analyzed using BioPharma Compass 3 (Bruker Daltonics). MS2 spectra were classified by ProteinScape using a HexNAc-HexNAc-Hex-Hex N-glycan core fragmentation pattern and subsequently subjected to glycan database searches using GlycoQuest (Bruker Daltonics) and protein database searches using MASCOT 2.5.1 (Matrix Science).

Using optimized low- and high-collision energies for respective glycan- and peptide-moiety fragmentation we performed measurements with combinations of increasing accumulation + ramp times with different MS2 target intensities for PASEF. The number of detected MS1 features that serve as precursors for PASEF steadily decreases with lower accumulation and ramp times due to the abundance distribution of glycoproteins in plasma. As a result the total number of acquired MS2 scales accordingly with increased accumulation + ramp times as more precursor are detected. The target PASEF intensity scales the number of replicate MS2 spectra recorded and summed for a single precursor according to its precursor intensity in MS1. Higher target PASEF intensities increase MS2 sensitivity but negatively impact the number of unique precursors that can be fragmented per duty cycle. Best results for plasma glycoproteomics were obtained by setting parameters that maximize the number of ions accumulated in the TIMS section and aim for high MS2 sensitivity.

Glycopeptide ID example: dominant glycoforms of hemopexin

```
10         20         30         40         50         60
MARVLGAPVA LGLWSLCWSL AIATPLPPTS AHGNVAEGET KPDPDVTERC SDGWSFDATT
70         80         90         100        110        120
LDDNGTMLFF KGEFVWKSHK WDRELISERW KNFPSPVDAA FRQGHNSVFL IKGDKVWVYP
130        140        150        160        170        180
PEKKEKGYPK LLQDEFPGIP SPLDAAVECH RGECQAEGVL FFQGDREWFW DLATGTMKER
190        200        210        220        230        240
SWPAVGNCSS ALRWLGRYYC FQGNQFLRFD PVRGEVPPRY PRDVRDYFMP CPGRGHGHRN
250        260        270        280        290        300
GTGHGNSTHH GPEYMRCSPH LVLSALTSDN HGATYAFSGT HYWRLDTSRD GWHSWPIAHQ
310        320        330        340        350        360
WPQGPSAVDA AFSWEEKLYL VQGTQVYVFL TKGGYTLVSG YPKRLEKEVG TPHGIILDSV
370        380        390        400        410        420
DAAFICPGSS RLHIMAGRRL WWLDLKSGAQ ATWTELPWPH EKVDGALCME KSLGPNSCSA
430        440        450        460        470
NGPGLYLIHG PNLYCYSDVE KLNAAKALPQ PQNVTSLLGC TH
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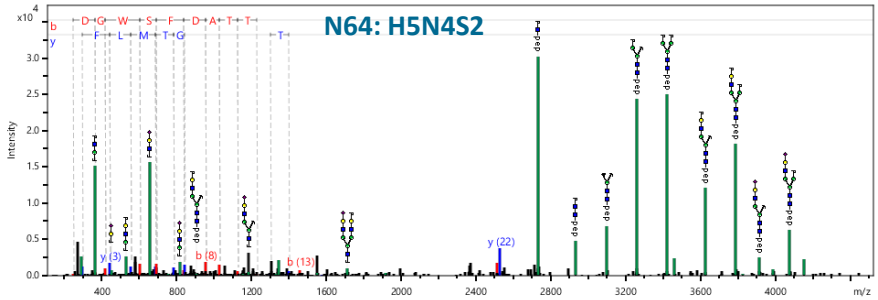
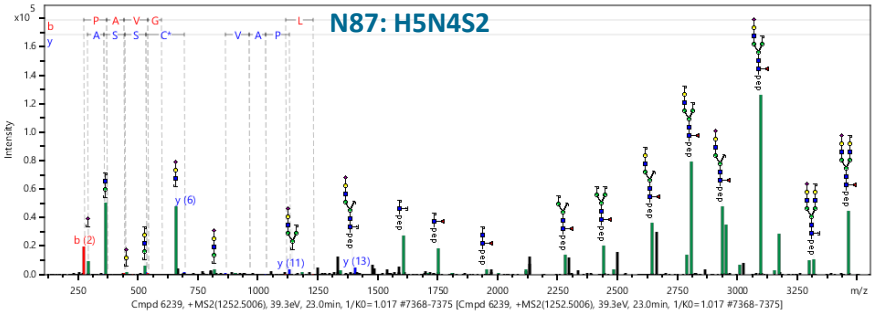
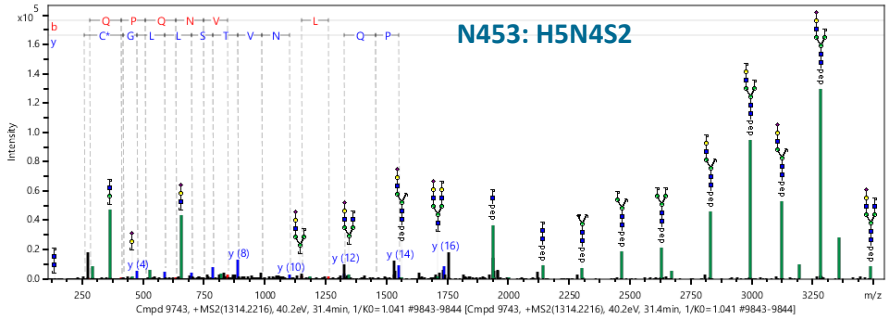
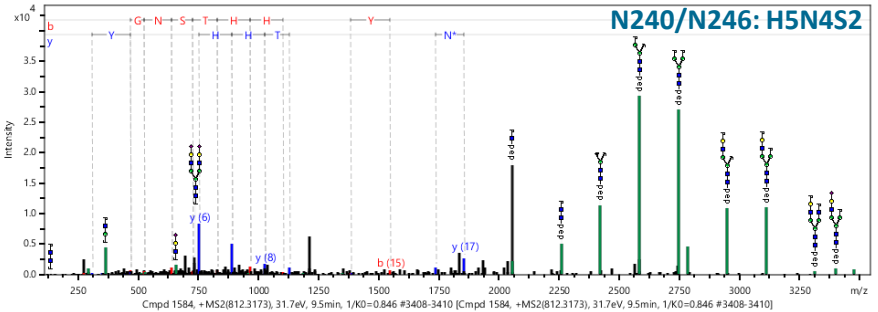








Peaks GlycoFinder: prototype 4D glycoproteomics software

Screenshot of the prototype PEAKS GlycoFinder user interface

PEAKS GlycoFinder is currently being developed by BioInformatics Solutions Inc. In collaboration with Bruker Daltonics and Radboudumc for comprehensive ion mobility aware glycoproteomics. The current prototype combines novel data processing with advanced glycan- and peptide-moiety identification strategies using both database searches and *de novo* sequencing for deep glycoproteome profiling.

4D feature detection for label-free quantitation combined with bioinformatic data processing enables users to explore protein glycobiology through intuitive graphical representations of the data from different perspectives.

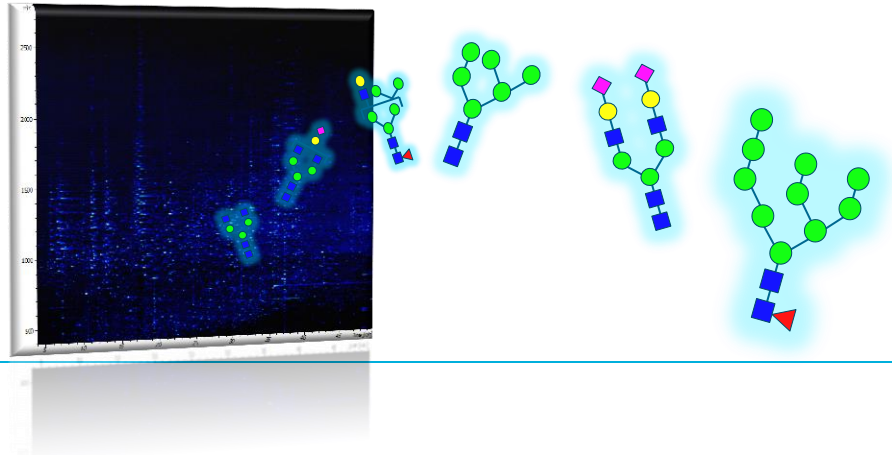
Preliminary results for a small subset of 6 full plasma glycopeptide data files acquired on a timsTOF Pro with optimized acquisition parameters yielded 2423 unique glycopeptides from 187 glycoproteins. Further optimization of algorithms is in progress to further improve glycopeptide identification results.

Conclusions

We have characterized and optimized critical parameters for succesful implementation of N-glycopeptide profiling in human plasma on the timsTOF Pro platform. Follow up work is now focussed on glycopeptide software development and application of PASEF glycopeptide technology in clinical applications to improve patient care.

Take home messages:

- Addition of TFA to mobile phase is required for analysis of highly sialylated glycoforms using ReproSil C18AQ2 stationary phase
- High TIMS delta potentials lead to in-TIMS fragmentation of glycopeptides
- nanoBooster N2 source gas dopants are compatible with TIMS, significantly increase sensitivity, and shift charge state distributions
- Best plasma N-glycopeptide identification results are obtained with PASEF parameters that take advantage of the timsTOF Pro MS/MS acquisition speed to maximize sensitivity
- Application of our optimzed PASEF workflow in combination with prototype 4D glycoproteomics software enables the analysis of thousands of N-glycopeptides in unfractionated human plasma samples



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