The Fine Art of Destruction for In-Depth Mass Spectrometry-Based Glycoproteomics:

Advances in Measurement and Data Analysis — Exploiting the Diagnostic Potential of Fragment Ions

Dr. Erdmann Rapp

Head of Bio/Process Analytics
Max Planck Institute
for Dynamics of Complex Technical Systems
Magdeburg, Germany

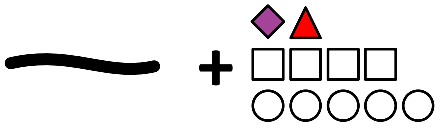
May 25, 2022 | Lisbon, Portugal

Towards Structural Glycoproteomics

Glycoproteomics

Highest Level of Detail and Confindence

LC-MS: Increased evidence based on precursor and fragment ions

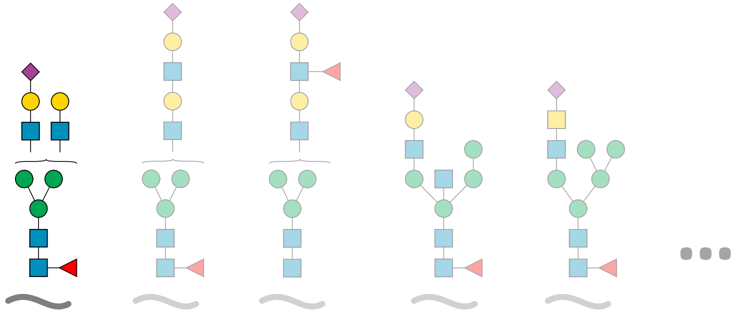


Peptide Seq.

Glycan Mass

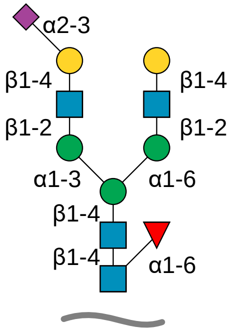
implicit

Peptide + $HexNAc_2Hex_5Fuc_1NeuAc_1$



Most likely Structure

Alternative Isobaric Structures



Actual Fine Structure

Glycan Composition

+Topology

Uncertainty

+Connectivity

+Configuration

Hexoses (Hex) ● Glc ● Gal ● Man

N-acetyl-hexosamine (HexNAc) ■ GlcNAc ■ GalNAc

Desoxyhexose ▲ Fuc

Sialic acid ◆ NeuAc

Examples of Conflicting Masses in Glycoproteomics

NeuAc	291.0954	
2x Fucose	292.1158	
Δ	1.0204*	

NeuGc	307.0903	
Hexose + Fucose	308.1107	
Δ	1.0204*	

NeuGc + Fucose	453.1482	
NeuAc + Hexose	453.1482	
Δ	≈ 0	(1.63×10^{-6})

Carbamidomethylation	57.0215	
Glycine	57.0215	
Δ	0	

Fucose + Carbamidomethyl./Glycine	203.0794	
HexNAc	203.0794	
Δ	≈ 0	(1.11×10^{-7})

Fucose + Oxidation on Peptide/F→Y	162.0528	
Hex	162.0528	
Δ	≈ 0	(1.00×10^{-6})

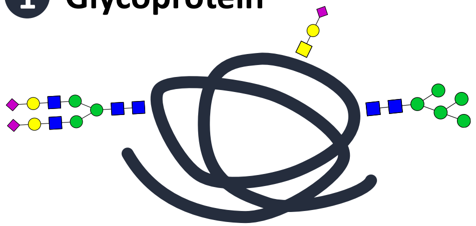
HexNAc + Oxidation on Peptide/F→Y	219.0743	
Hex + Carbamidomethyl./Glycine	219.0743	
Δ	≈ 0	(8.89×10^{-7})

Masses and mass differences in Da.

*conflicts for instance with N deamidation (Δ 0.985 Da)

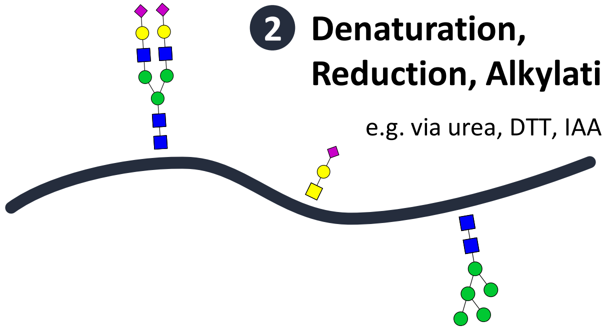
Generic Glycoproteomics Workflow

① Glycoprotein

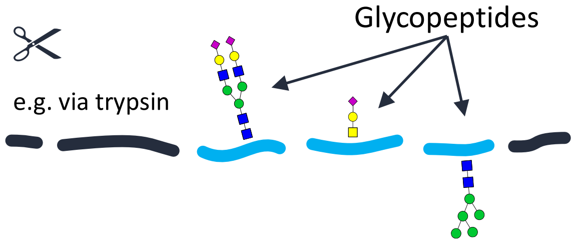


② Denaturation, Reduction, Alkylation

e.g. via urea, DTT, IAA



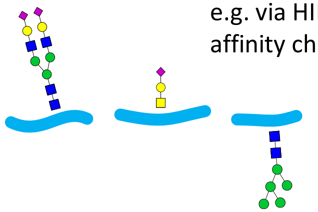
③ Proteolytic digestion



e.g. via trypsin

④ Glycopeptide enrichment

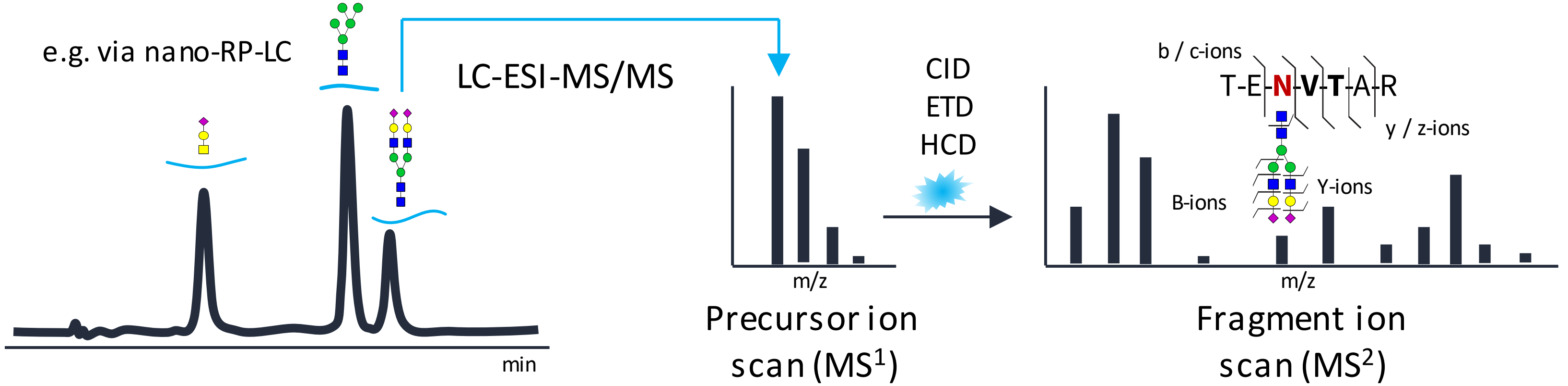
e.g. via HILIC-SPE, lectin affinity chromatography



⑤ Chromatographic separation

⑥ Mass spectrometric analysis

e.g. via nano-RP-LC



Precursor ion scan (MS^1)

Fragment ion scan (MS^2)

⑦ Data Analysis

Glycopeptide Identification & characterization

- ○ Sites
- ○ Micro- & macroheterog.

N- and *O*-Glycoproteomics Workflow

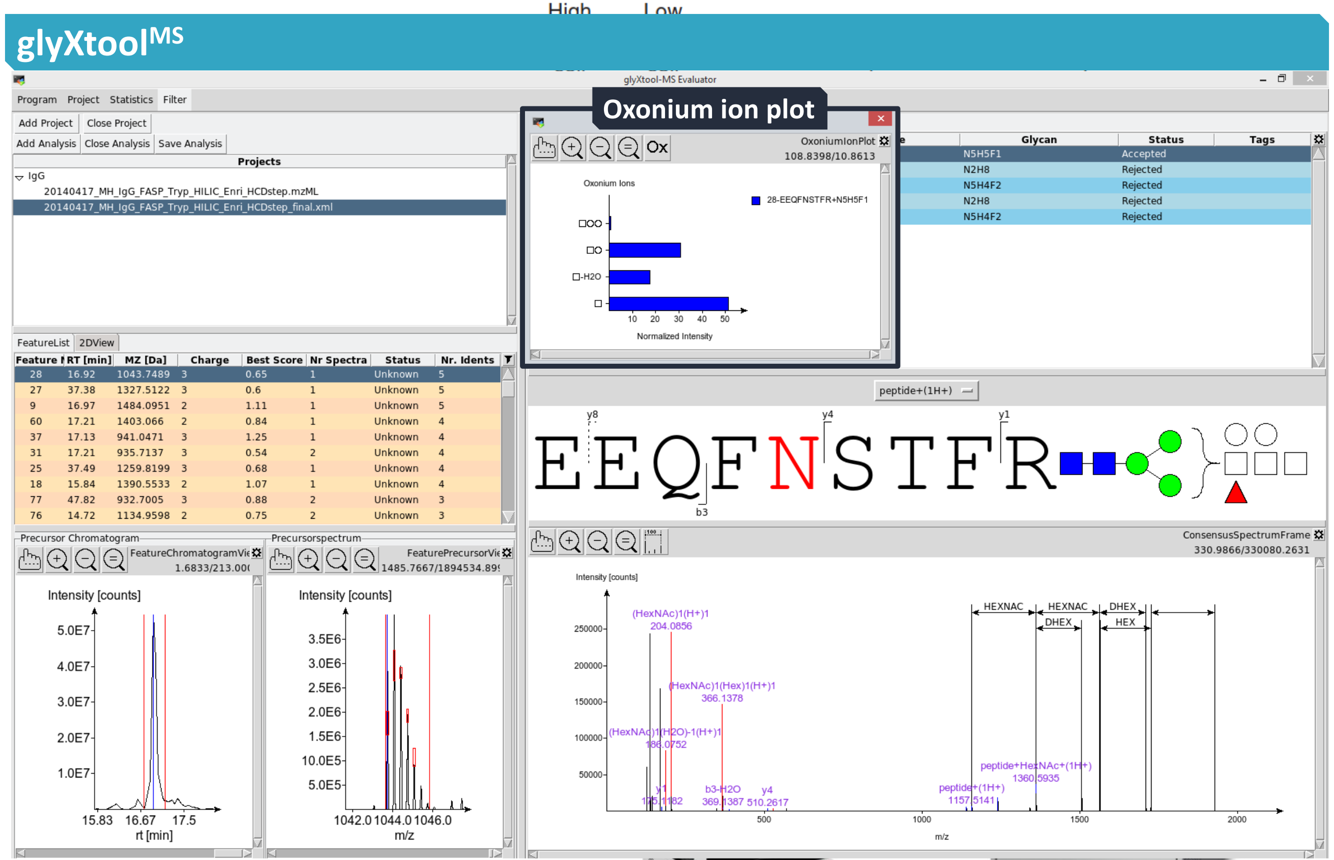
Exemplary Glycopro
Human: Immunoglobulin
Bovine: Ribonuclease B

FASP Digest
10 kDa MWCO

Glycopeptid
Enrichment

nanoRP-LC-E
OT-OT MS/M

Data Processing/
Manual Validation



glyXtool^{MS}

glyXtool-MS Evaluator

Projects:
- IgG
  - 20140417_MH_IgG_FASP_Tryp_HILIC_Enri_HCDstep.mzML
  - 20140417_MH_IgG_FASP_Tryp_HILIC_Enri_HCDstep_final.xml

Feature I	RT [min]	MZ [Da]	Charge	Best Score	Nr Spectra	Status	Nr. Idents
28	16.92	1043.7489	3	0.65	1	Unknown	5
27	37.38	1327.5122	3	0.6	1	Unknown	5
9	16.97	1484.0951	2	1.11	1	Unknown	5
60	17.21	1403.066	2	0.84	1	Unknown	4
37	17.13	941.0471	3	1.25	1	Unknown	4
31	17.21	935.7137	3	0.54	2	Unknown	4
25	37.49	1259.8199	3	0.68	1	Unknown	4
18	15.84	1390.5533	2	1.07	1	Unknown	4
77	47.82	932.7005	3	0.88	2	Unknown	3
76	14.72	1134.9598	2	0.75	2	Unknown	3

Glycan	Status	Tags
N5H5F1	Accepted	
N2H8	Rejected	
N5H4F2	Rejected	
N2H8	Rejected	
N5H4F2	Rejected	

glyXtoolMS

Co-developed with glyXera, open access, open source [2]

Ultimate 3000 RSLCnano
UHPLC System

LTQ Orbitrap Elite
hybrid mass spectrometer

FASP, Filter Aided Sample Prep. *SPE, Solid-phase extraction* *HCD, Higher-energy collisional dissociation* *MWCO, Molecular Weight Cut-Off*

[1] Selman et al., Anal Chem. 2011

[2] Pioch et al., Anal Chem. 2018

glyXera‘s High Performance Glycoanalysis System glyXboxCE

glyXboxCE is based on xCGE-LIF ⇨ Allowing to conduct glycoanalysis from high-throughput to in-depth mode

- Capillary Arrays with 4-96 capillaries in parallel ⇨ Real High-Throughput ⇨ Up to 450-times faster !!! ✓
- Patented Technology ⇨ Highest Reproducibility & Robustness ⇨ RSD t_{mig} < 0.1 % & runs 24/7 !!! ✓
- Separation by charge, mass & shape ⇨ Highest Separation Power ⇨ Up to 15-times better !!! ✓
- Laser Induced Fluorescence Detection ⇨ Highest Sensitivity ⇨ Up to 3750-times higher !!! ✓

Full Structural Elucidation:

Topologies: bisecting, (core-) fucosylation, branching, etc.

Linkages: α, β / 1-6, 1-4, etc.

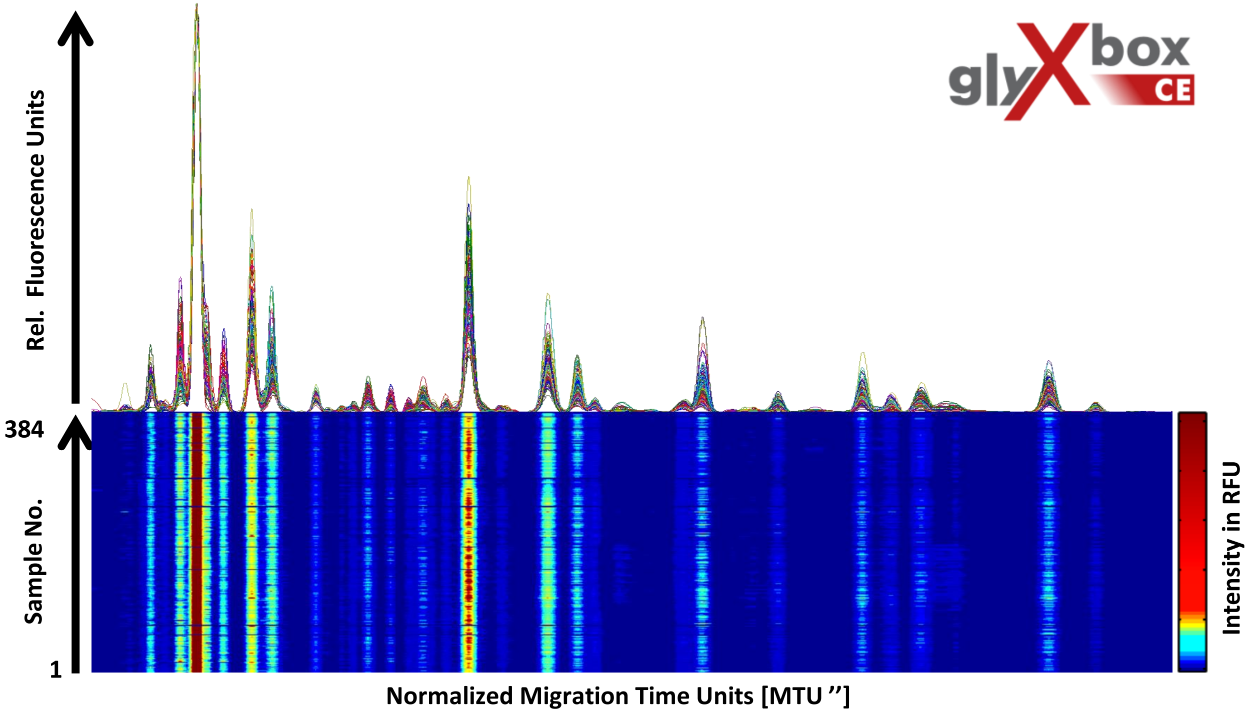
Monosaccharides:
● Glc, ● Man, ○ Gal, ■ GlcNAc, □ GalNAc, ▲ Fuc, ◆ NANA, ◇ NGNA

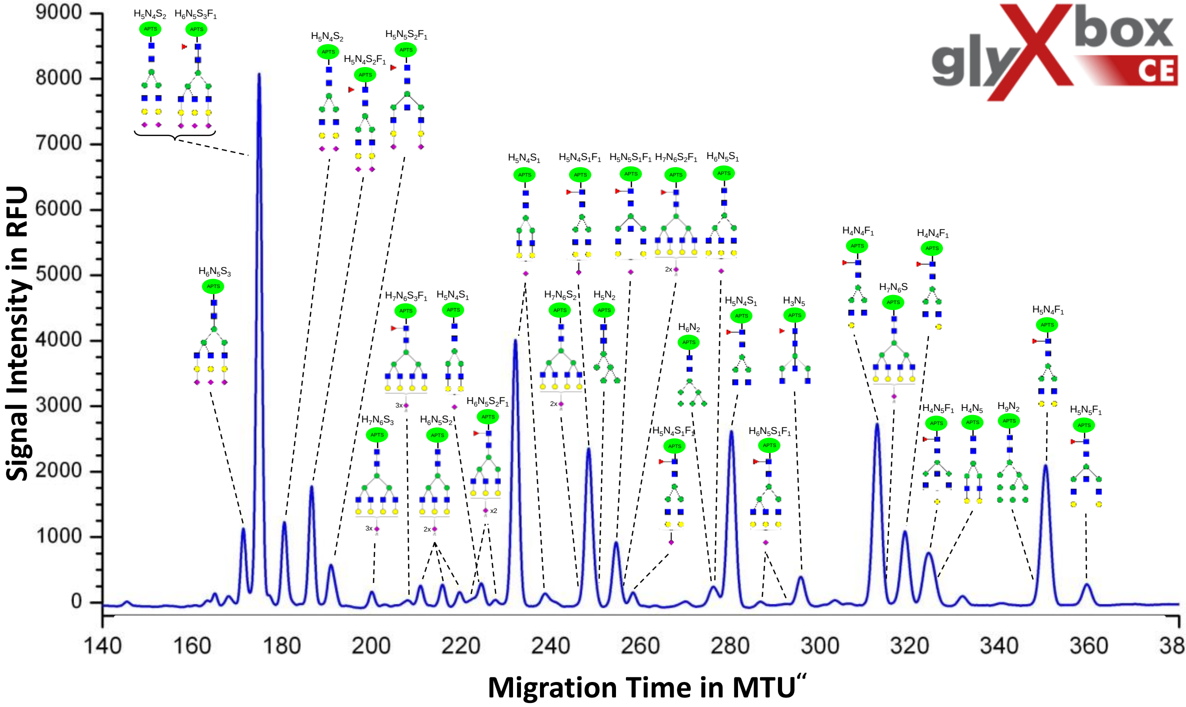
High Performance Glycoanalysis – Combining Expertise & Pace

Running glyXboxCE in Real High-Throughput Mode

glyXboxCE is based on xCGE-LIF

Standard Glycoprofiling ⇨ Database matching for structural assignment





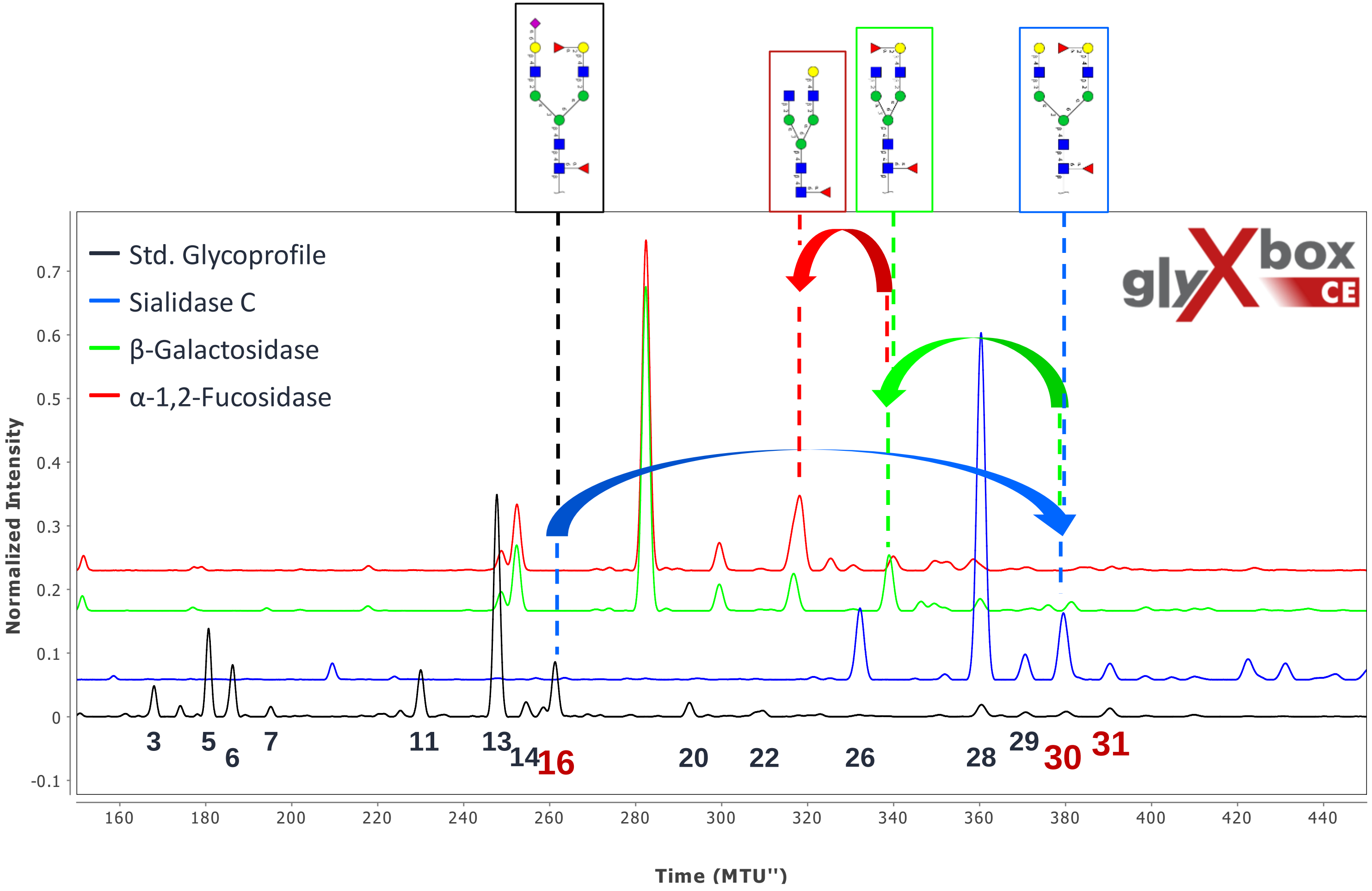
- **Separation of up to 4700 samples in 48 hours !!! ⇨ Automated Data Processing & Structural Assignment !!!** ✓

Running glyXboxCE in Real In-Depth Mode

glyXboxCE is based on xCGE-LIF

Extended Glycoprofiling ⇨ Exoglycosidase Sequencing for in-depth structural elucidation

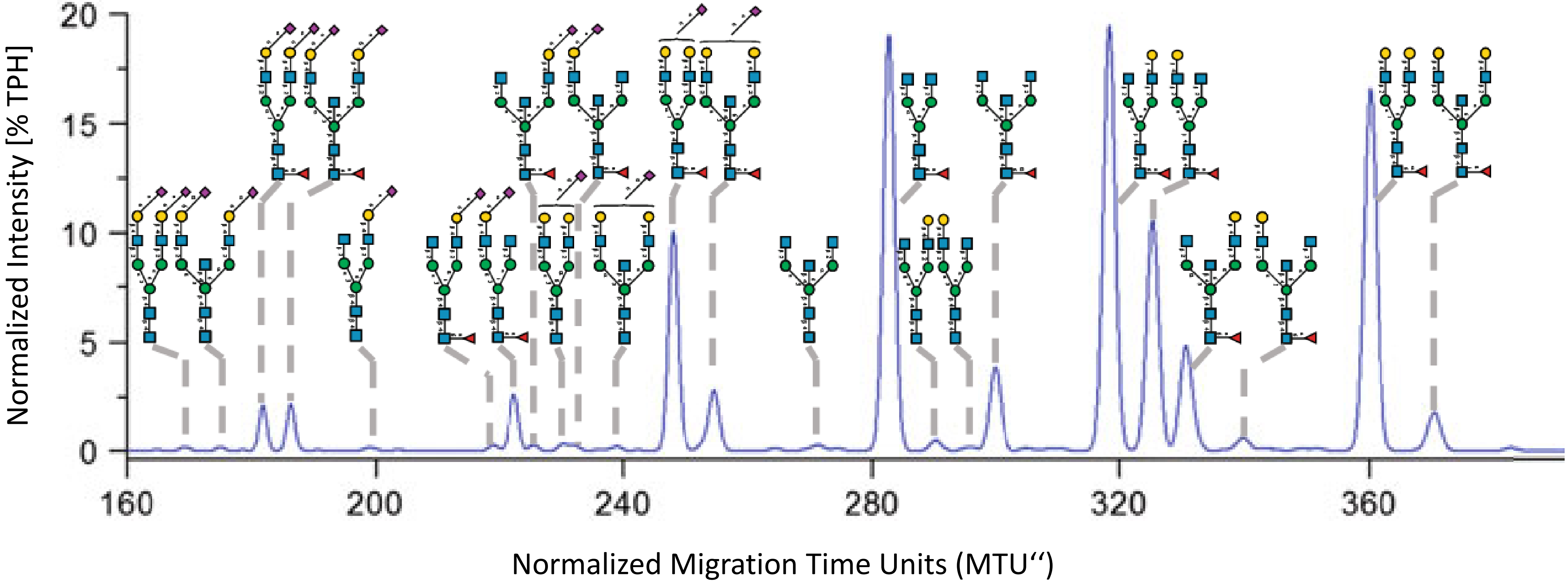
- **Parallel and/or Sequential Sequencing in Decent Time!!!** ✓
- **Full Structural Elucidation of New / Unknown Glycan-Peaks !!!** ✓
- **Elucidation of Composition of Multi-Structure Peaks !!!** ✓



N-Glycans: Full Structural Elucidation by glyXboxCE

Example: Human IgG *N*-Glycans *(Total N-Glycome)*

In-Depth *N*-Glycoprofiling (incl. Exocglycosidase Sequencing)

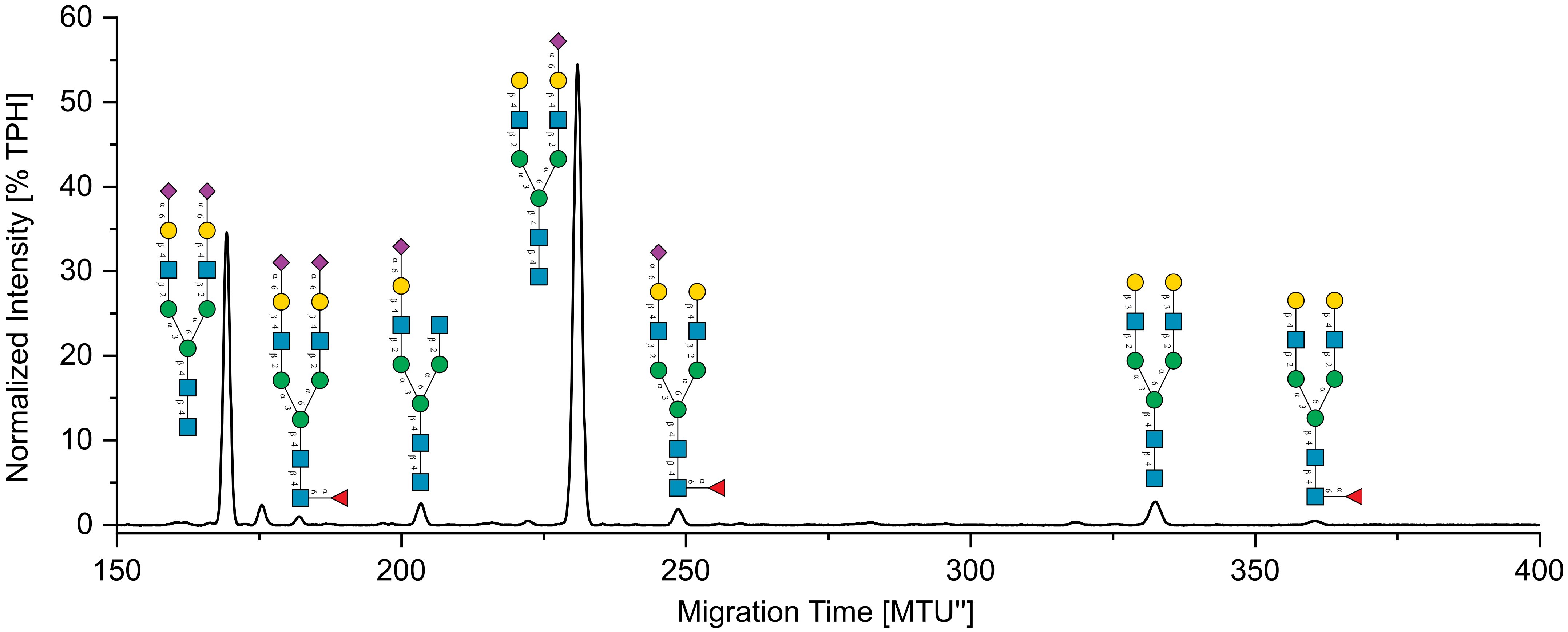


Cajic, S., et al. (2021). "Capillary (Gel) Electrophoresis-Based Methods for Immunoglobulin (G) Glycosylation Analysis." Experientia supplementum (2012) 112: 137-172.

TPH, total peak height

N-Glycans: Full Structural Elucidation by glyXboxCE

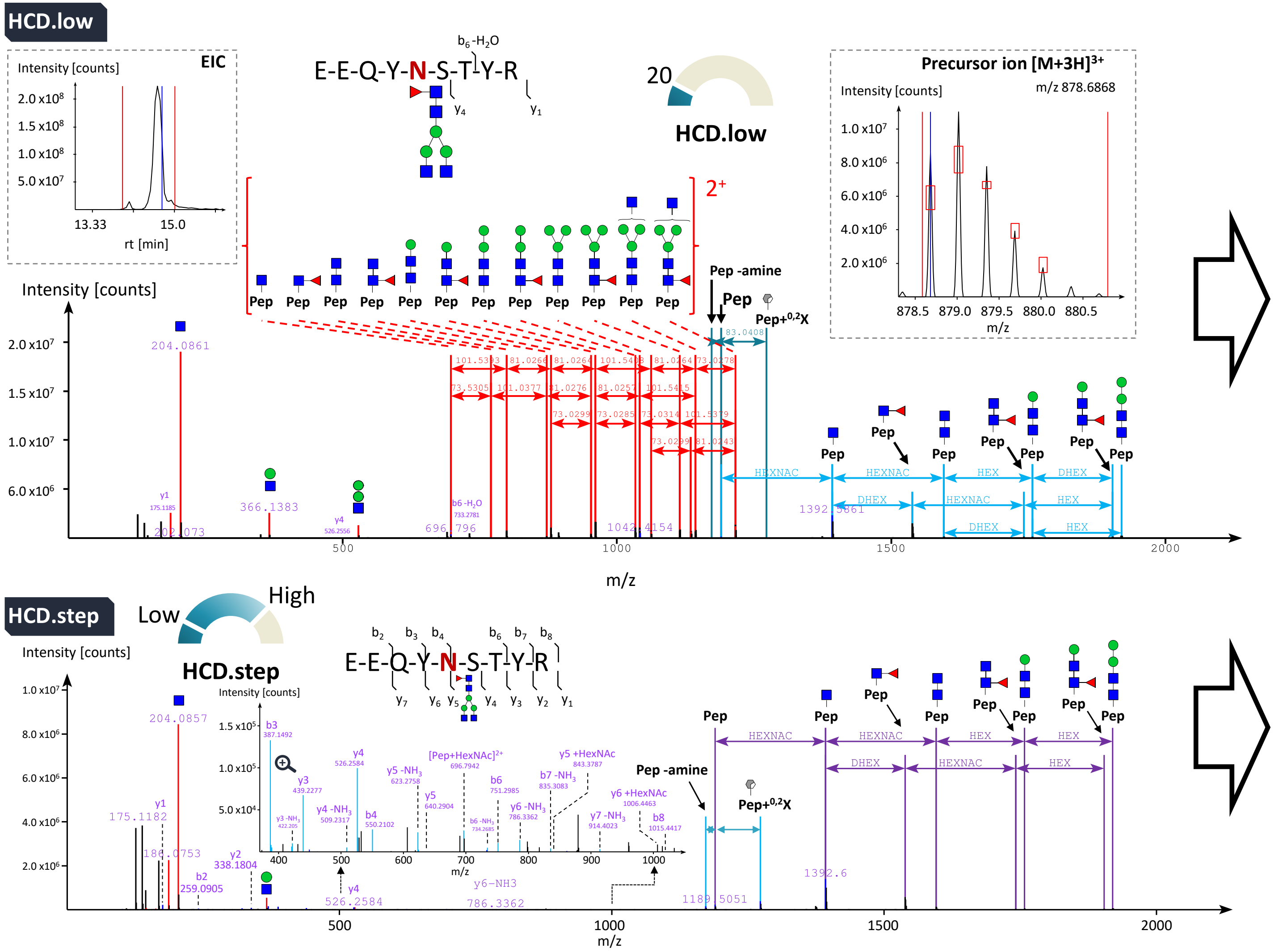
Example: Human Fibrinogen *N*-Glycans



Data kindly provided by V. Grote (MPI-Magdeburg)

TPH, total peak height

HCD Fragmentation of Glycopeptides



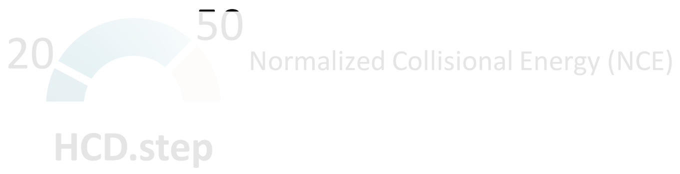
Identification and characterization of the ***N*-glycan moiety**, *incl. elucidation of structural features such as antenna- vs core fucosylation*

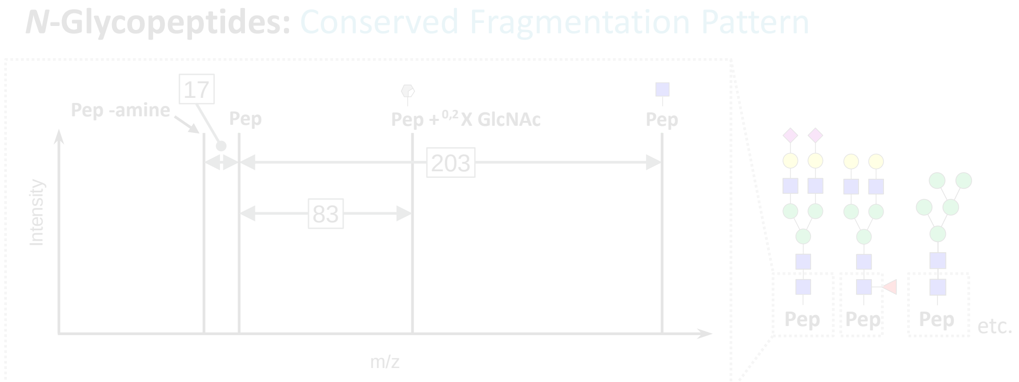
Identification of the **peptide moiety**

In-Depth N-Glycoproteomics

HCD Fragmentation of Glycopeptides

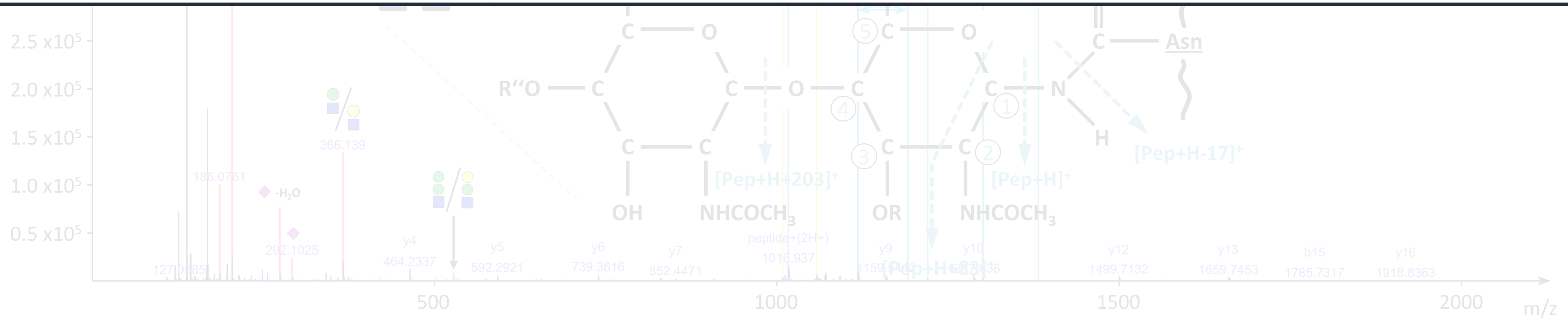
Conserved Fragmentation Pattern





Increased confidence in the correct annotation of the peptide mass of *N*-glycopeptides

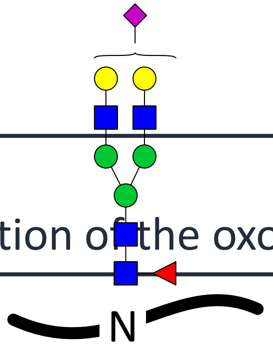
→ Relevance: Proteinase K digest, peptide mass cannot be predicted *a priori*

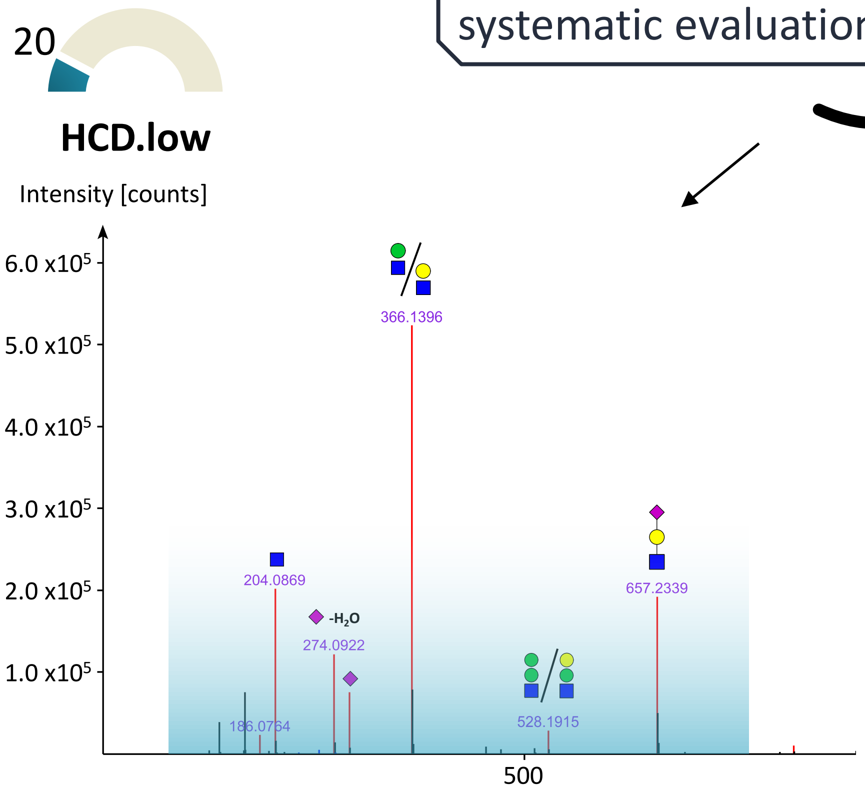


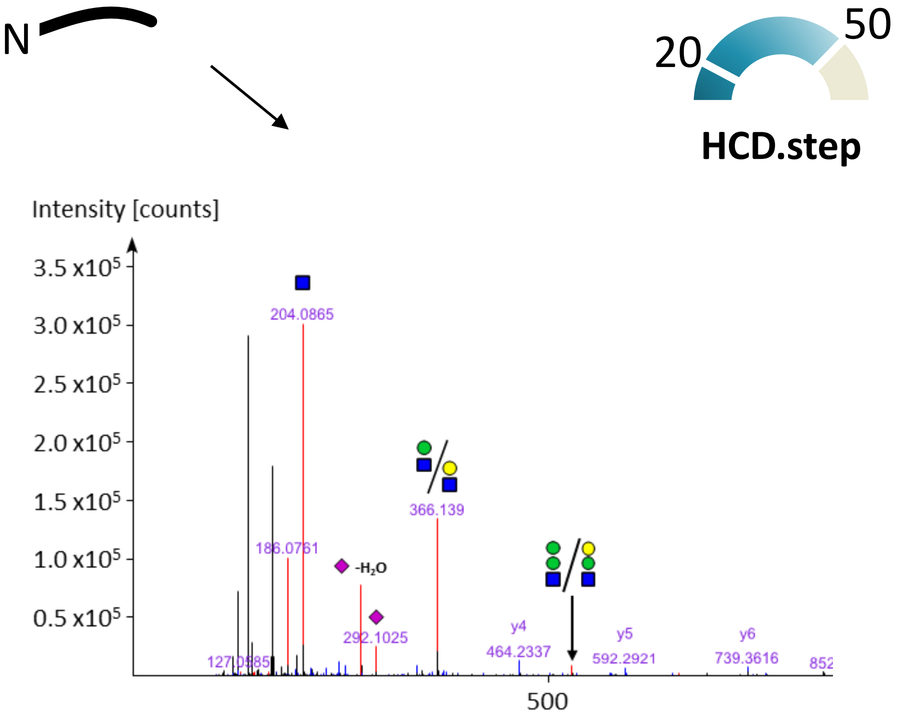
Oxonium Ions

Oxonium Ions Spectra Classification

Motivation:
systematic evaluation of the oxonium ion pattern

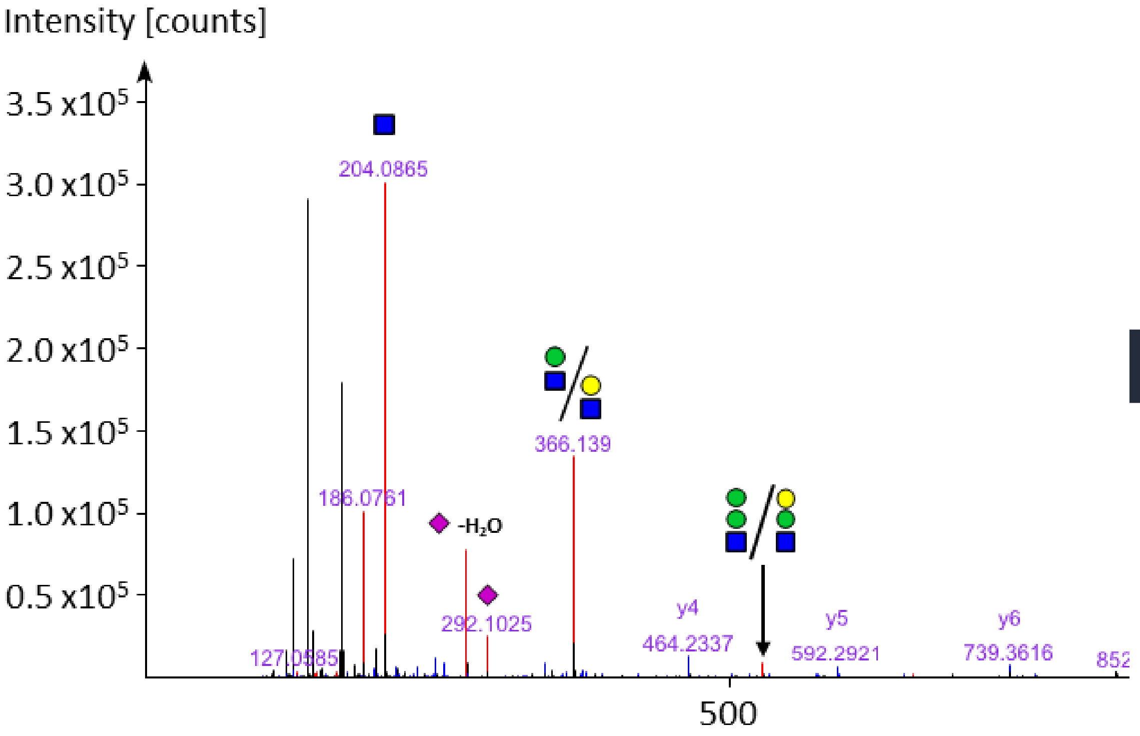




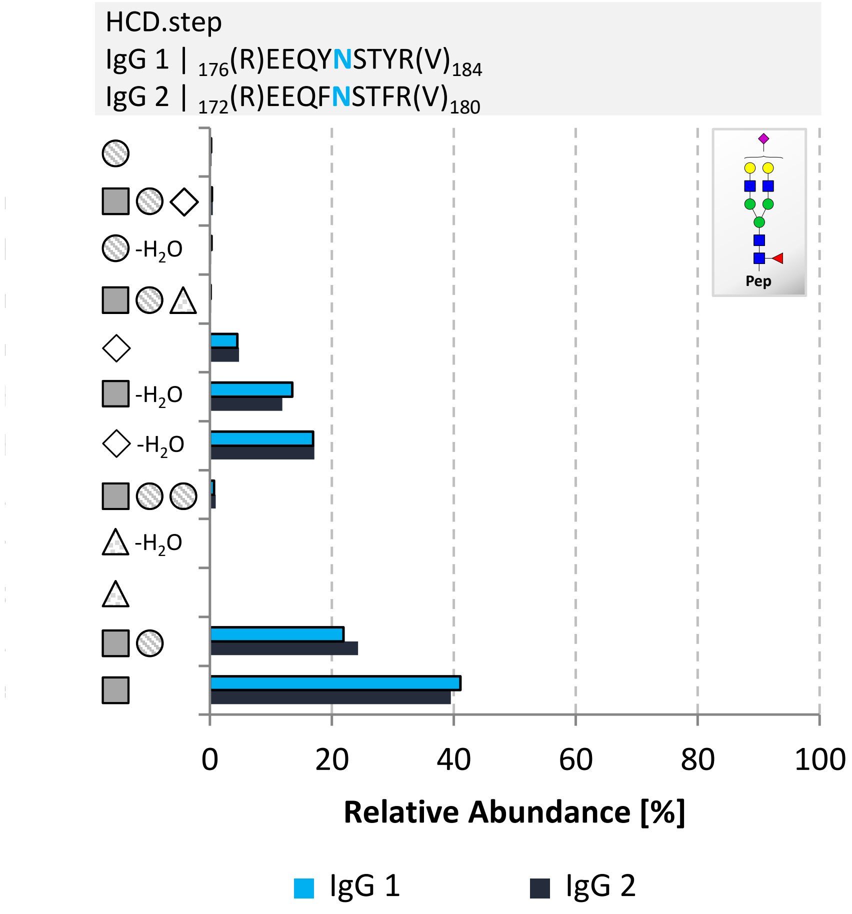


Oxonium Ion Quantitation

N-Glycopeptide Oxonium Ion Pattern



Bar Graph: Relative Oxonium Ion Abundance


HCD.step

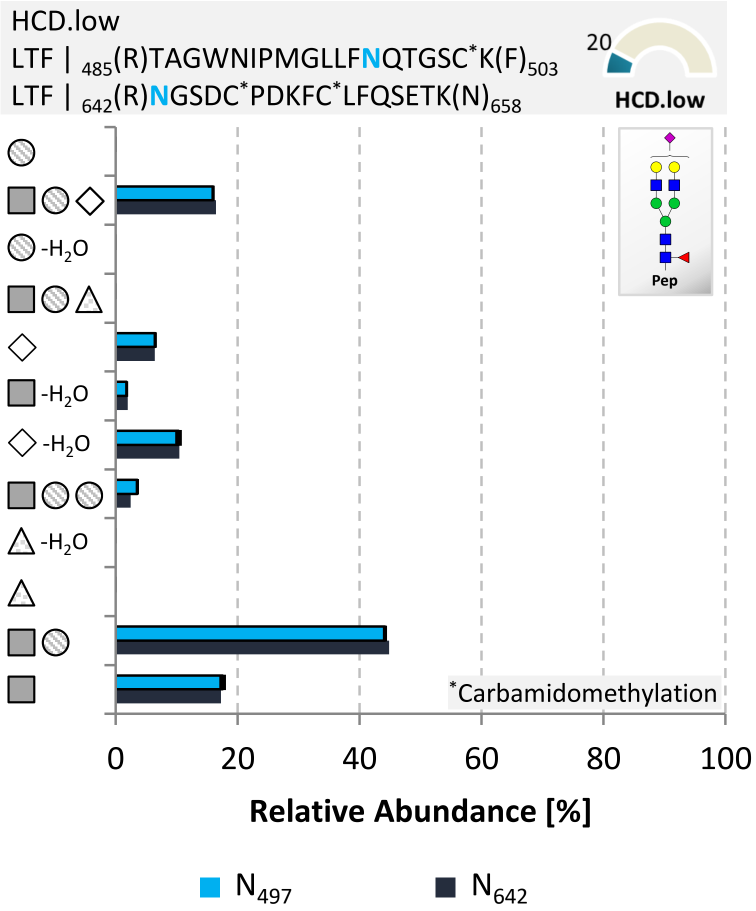
IgG 1 | $_{176}$(R)EEQY**N**STYR(V)$_{184}$

IgG 2 | $_{172}$(R)EEQF**N**STFR(V)$_{180}$

Oxonium Ions: Glycosylation Sites

N-Glycopeptide Oxonium Ion Pattern

- Independent of the glycosylation site ✓

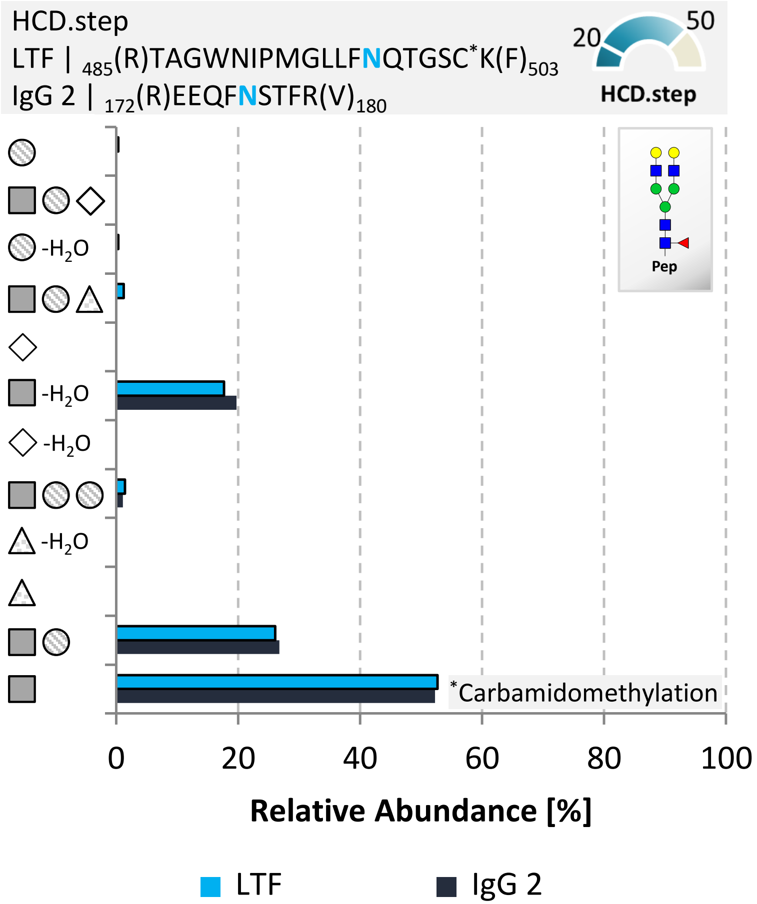


LTF - Lactotransferrin

Oxonium Ions: Peptide Backbone

N-Glycopeptide Oxonium Ion Pattern

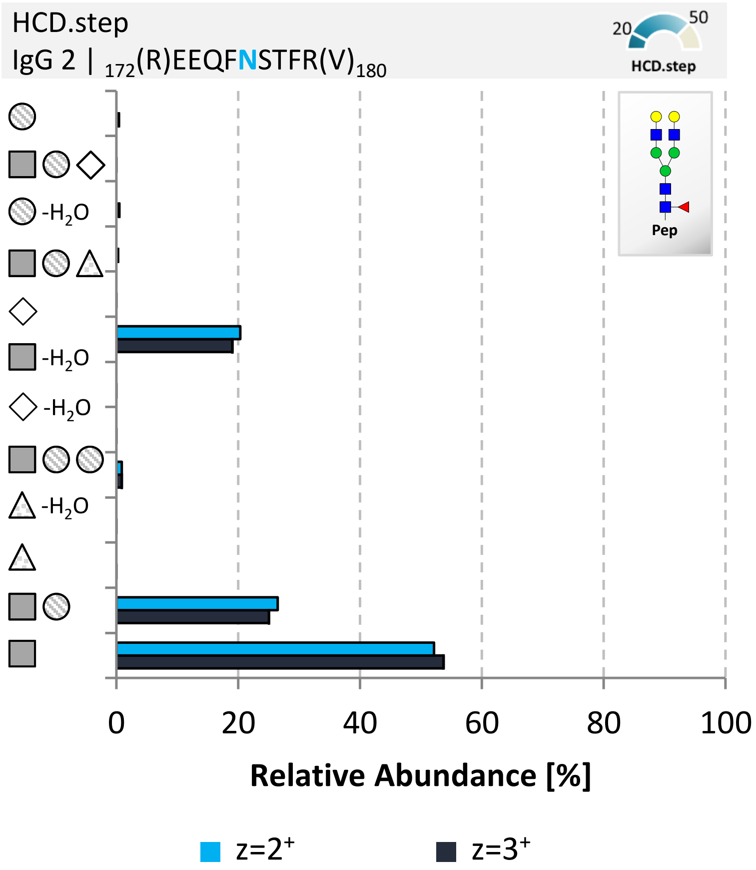
- Independent of the peptide backbone ✓



Oxonium Ions: Charge State of the Precursor Ion

N-Glycopeptide Oxonium Ion Pattern

- Independent of the charge state of the precursor ion ✓



Oxonium Ions

N-Glycopeptide Oxonium Ion Pattern

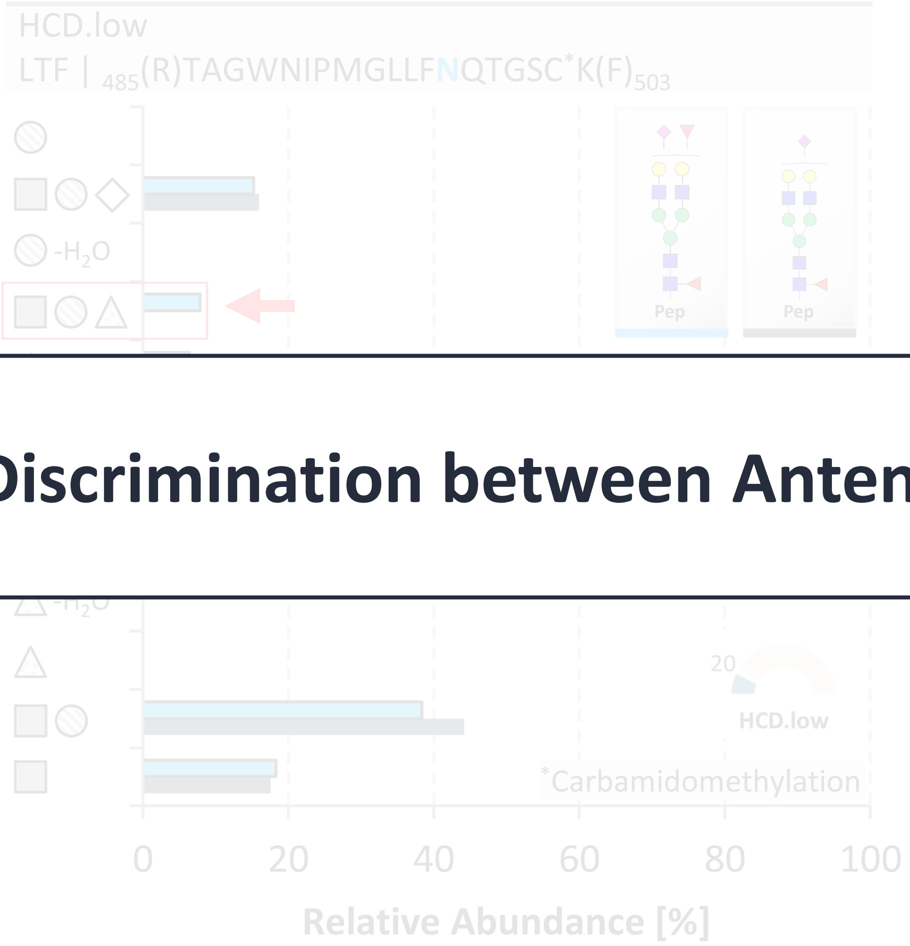
- Independent of the glycosylation site ✓
- Independent of the peptide backbone ✓
- Independent of the charge state of the precursor ion ✓
- Identical for the same glycan moiety ✓

Oxonium ion pattern directly reflect characteristics of the glycan moiety

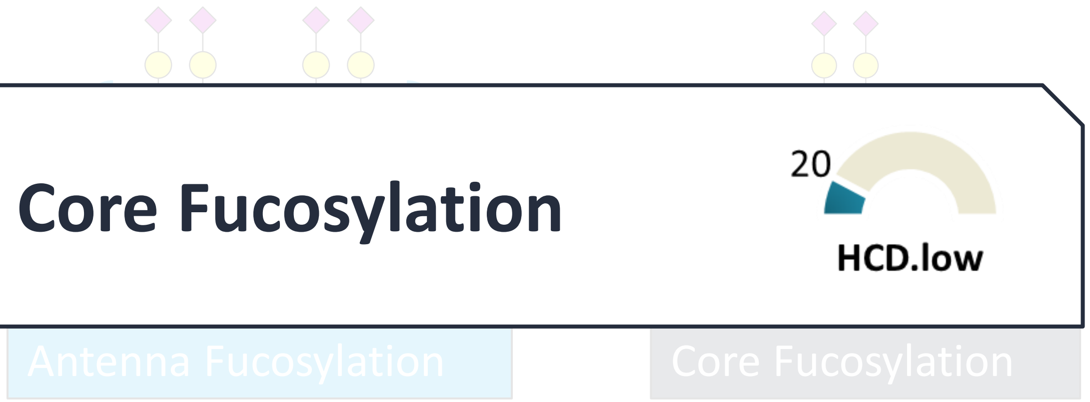
To what extend do oxonium ions provide structural glycan information?

Oxonium Ions: Core vs Antenna Fucosylation

N-Glycopeptide Oxonium Ion Pattern



Isobaric *N*-Glycan Structures



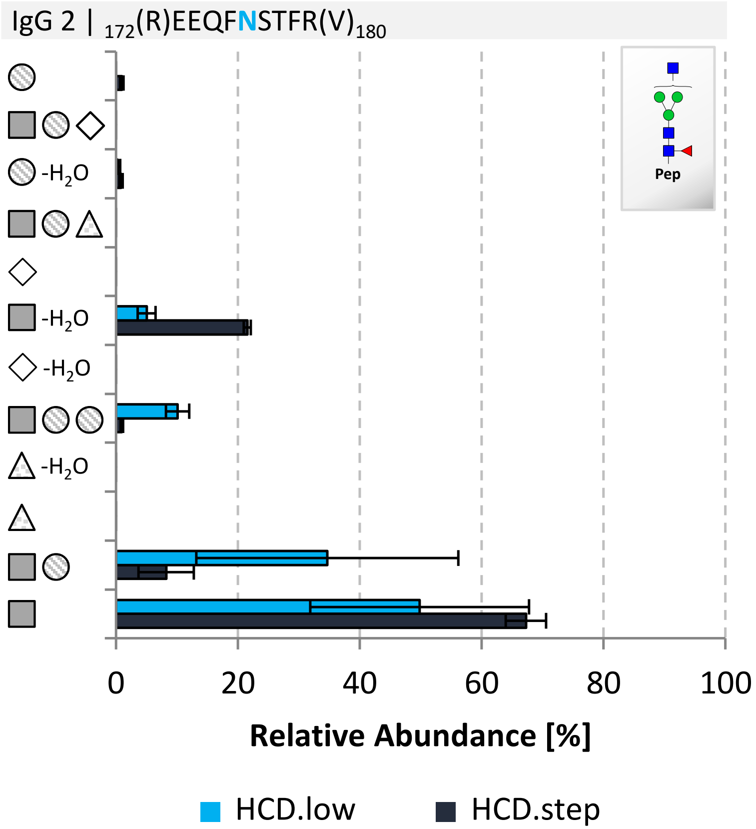
✓

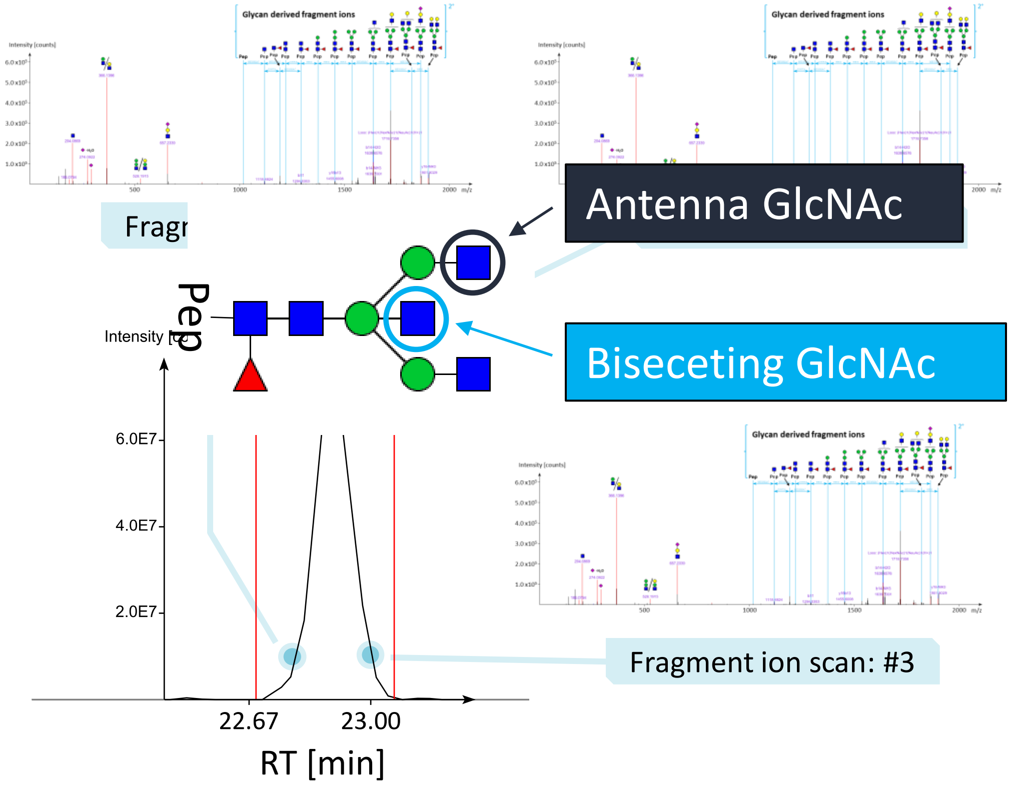
Discrimination between Antenna and Core Fucosylation

20
HCD.low

Oxonium Ions: Bisecting GlcNAc vs Antenna GlcNAc

N-Glycopeptide Oxonium Ion Pattern

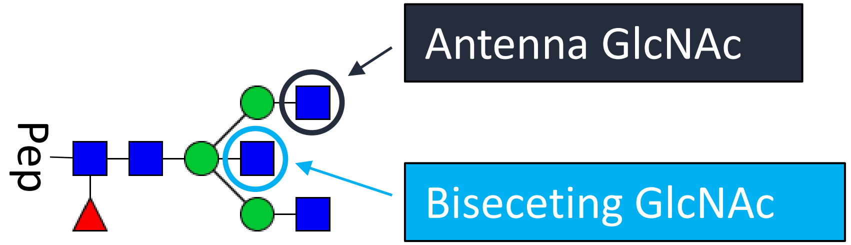


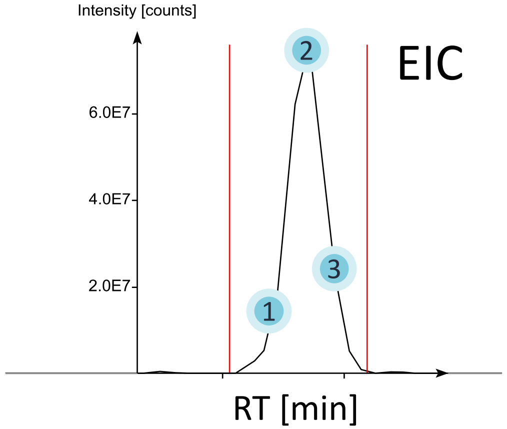


EIC, extracted ion chromatogram

Oxonium Ions: Bisecting GlcNAc vs Antenna GlcNAc

N-Glycopeptide Oxonium Ion Pattern





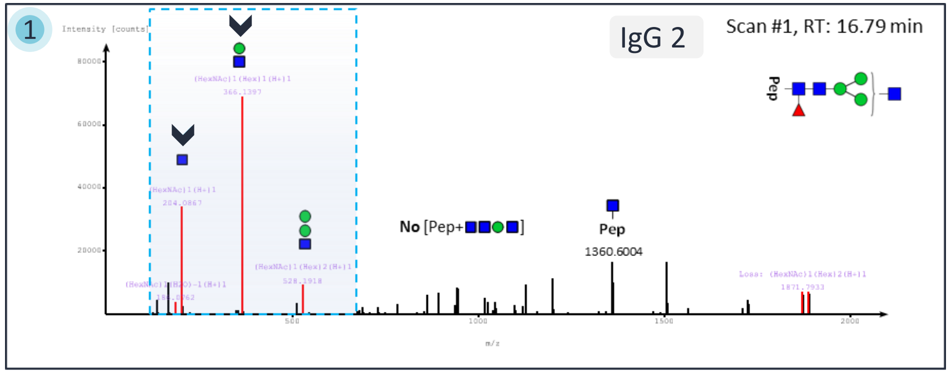
Antenna GlcNAc

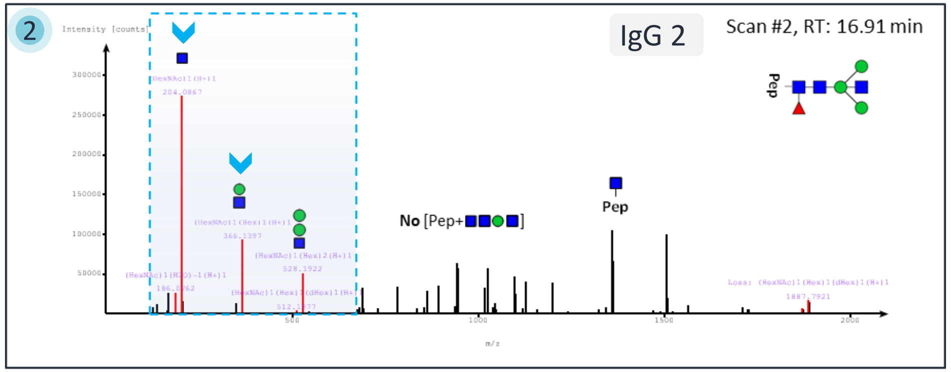
Oxonium ion pattern changes

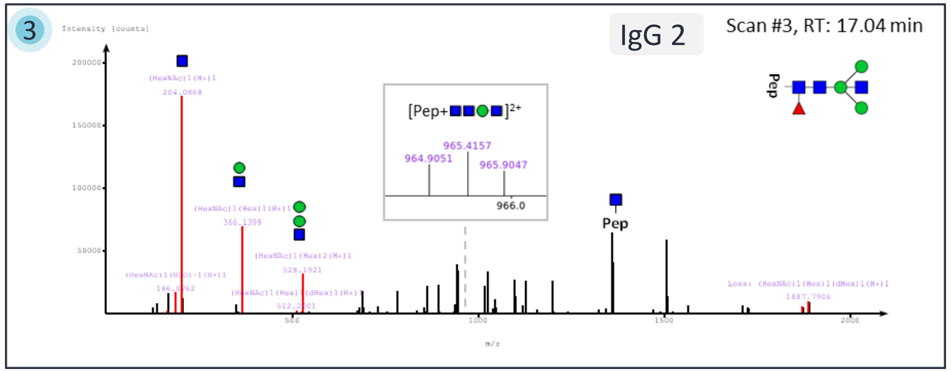
Bisecting GlcNAc

20

HCD.low

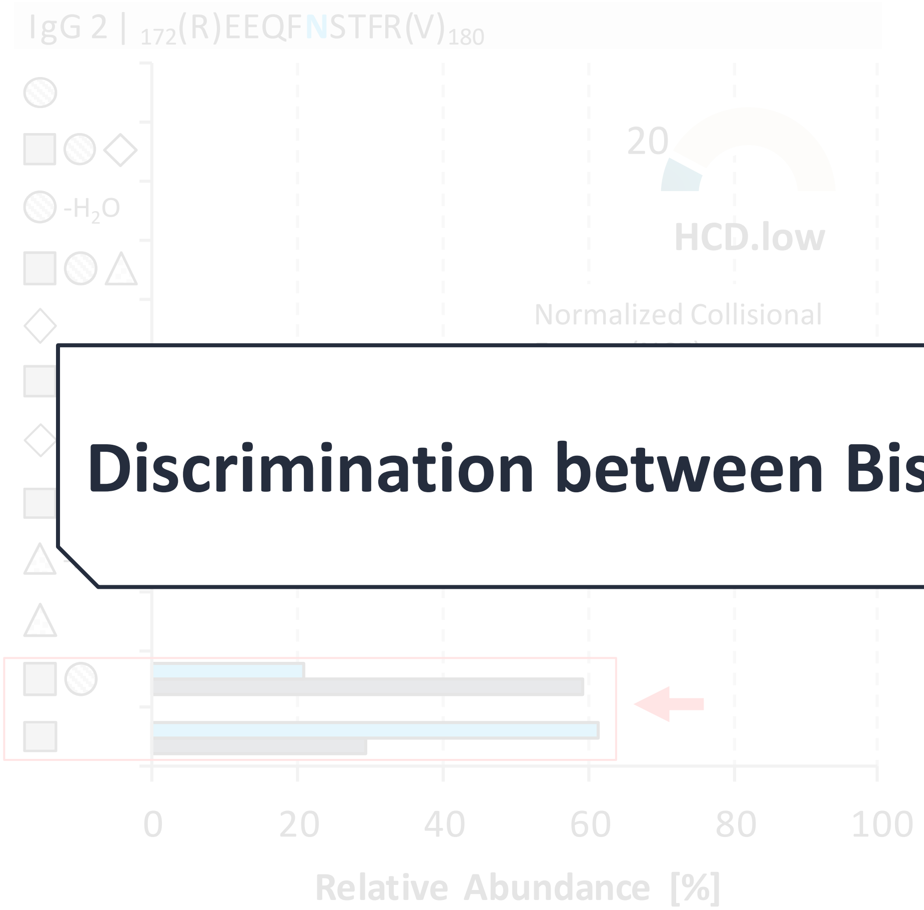




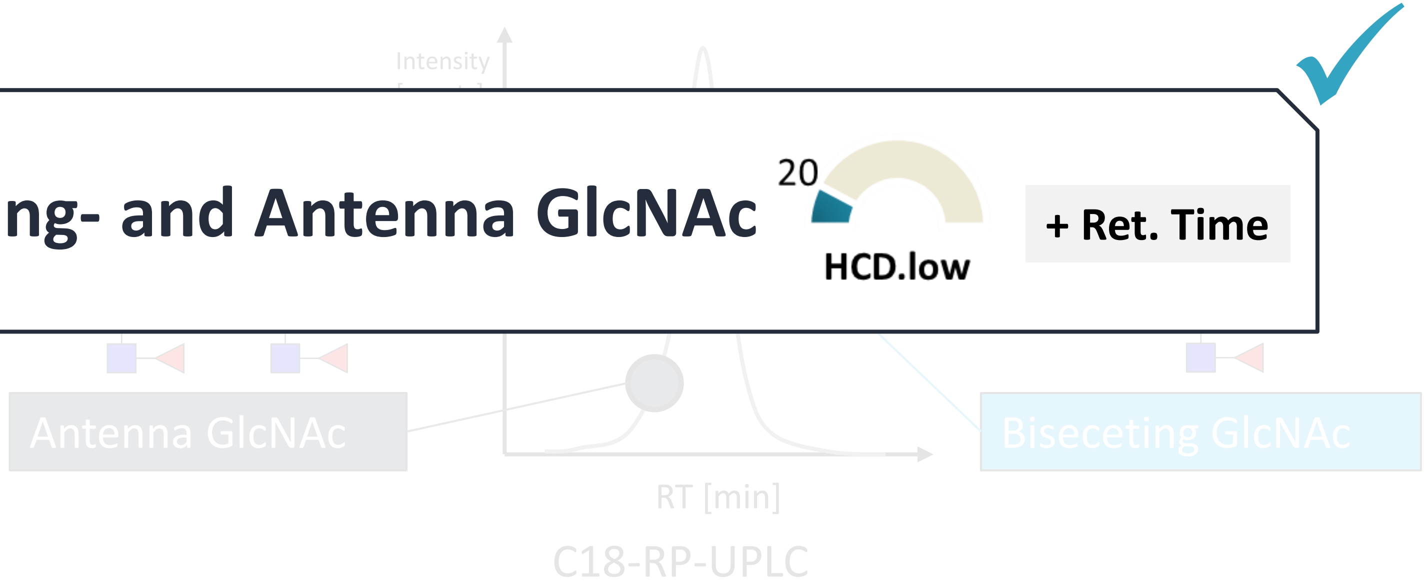


Oxonium Ions: Bisecting GlcNAc vs Antenna GlcNAc

N-Glycopeptide Oxonium Ion Pattern



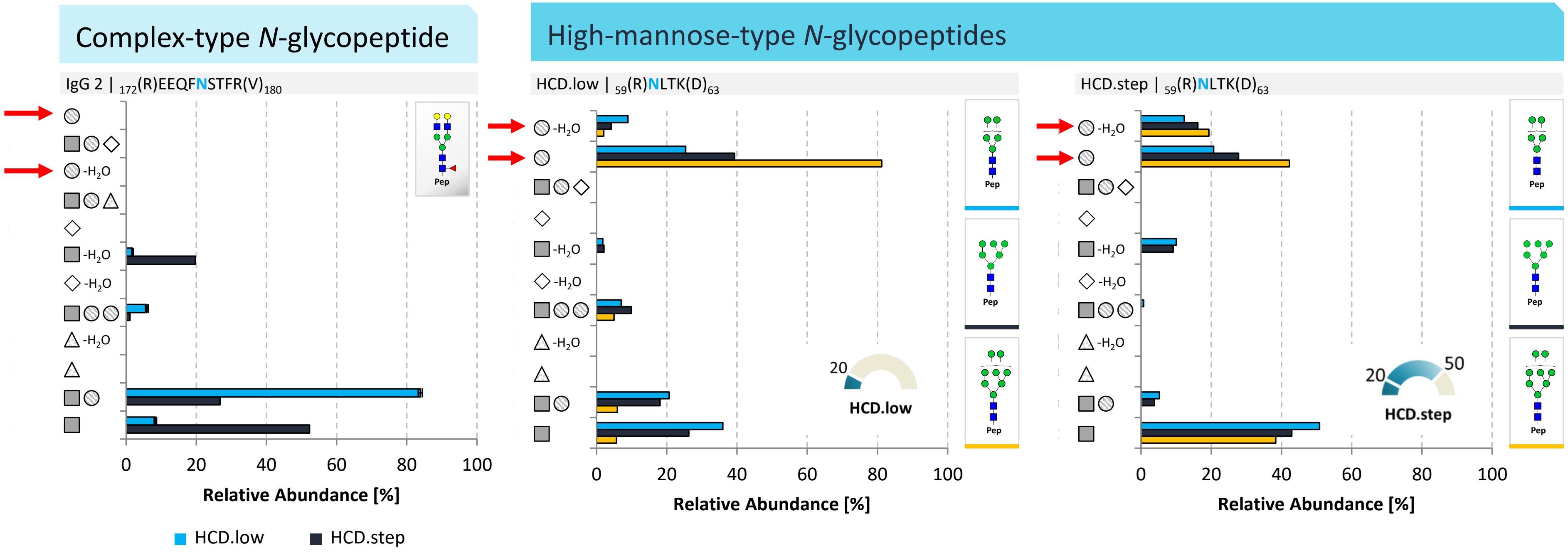
Isobaric *N*-Glycan Structures



Discrimination between Bisecting- and Antenna GlcNAc 20 HCD.low **+ Ret. Time**

Oxonium Ions: High-Mannose-Type *N*-Glycopeptides

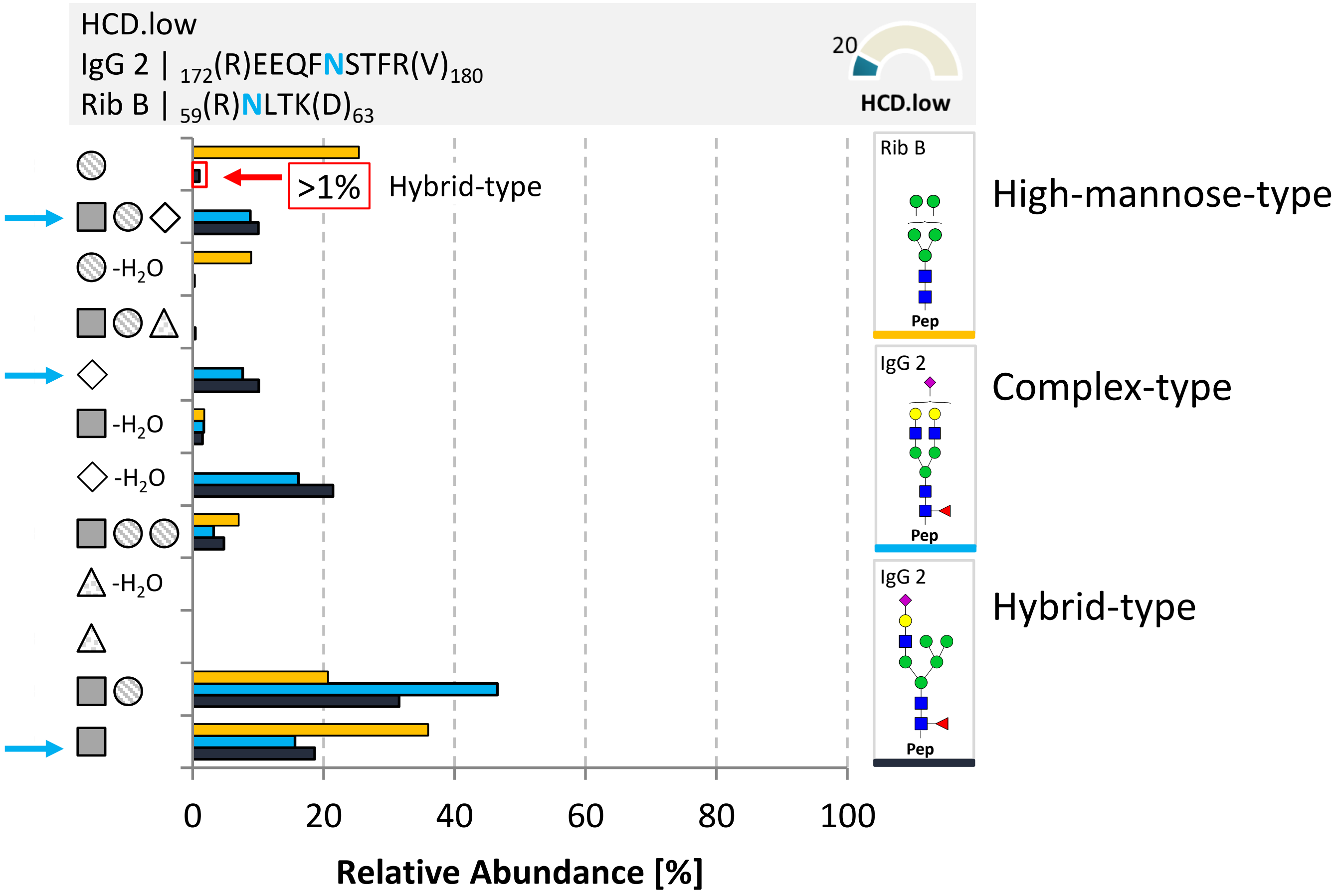
N-Glycopeptide Oxonium Ion Pattern



- HCD.low and HCD.step: Hex ▨ oxonium ion abundance correlates with number of mannoses ●
- Hex oxonium ions normally absent or hardly present (< 0.5%) with complex-type N-glycopeptides

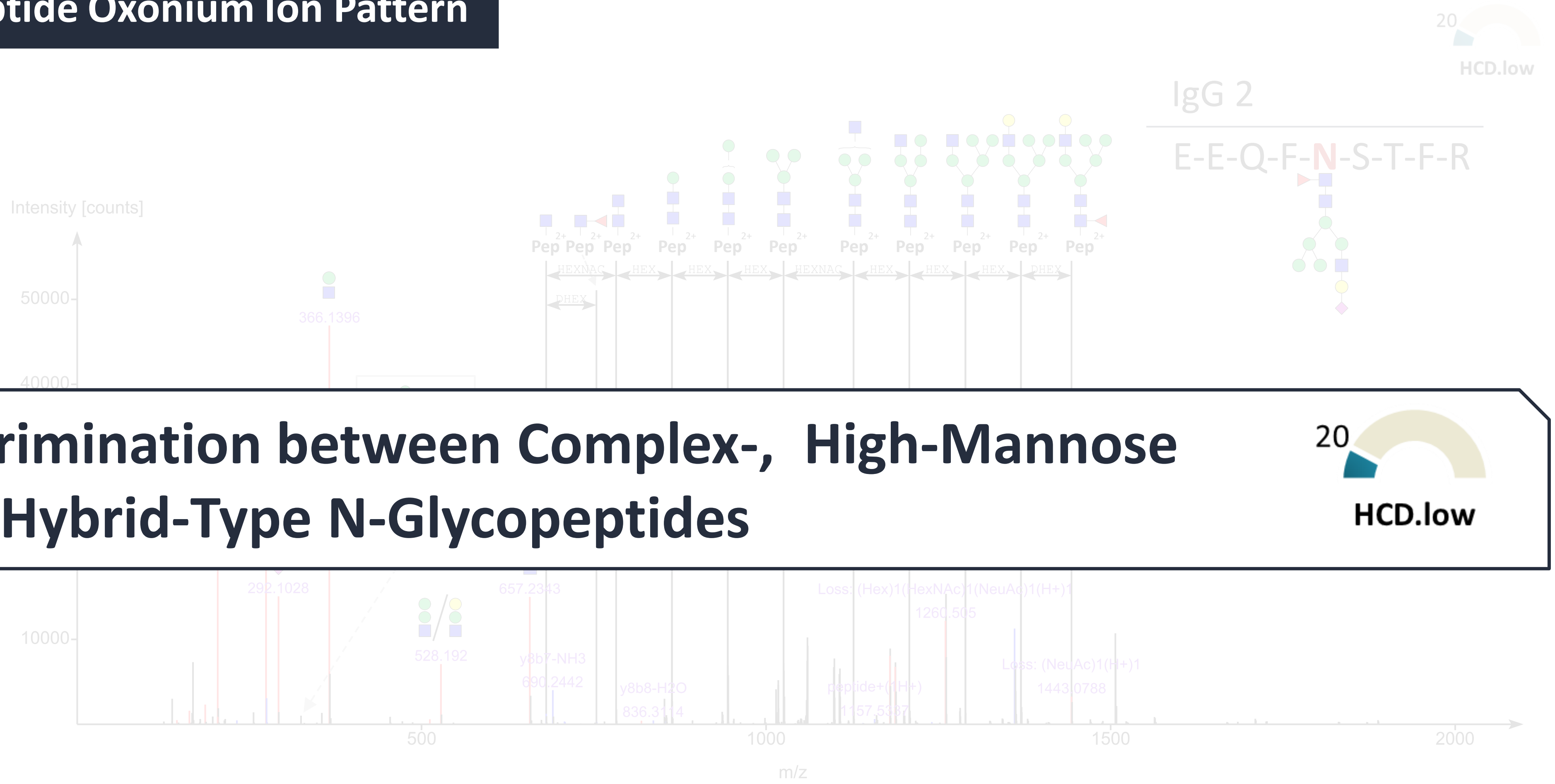
Oxonium Ions: Hybrid-Type *N*-Glycopeptides

N-Glycopeptide Oxonium Ion Pattern



Oxonium Ions: Hybrid-Type *N*-Glycopeptides

N-Glycopeptide Oxonium Ion Pattern



Discrimination between Complex-, High-Mannose and Hybrid-Type N-Glycopeptides

20

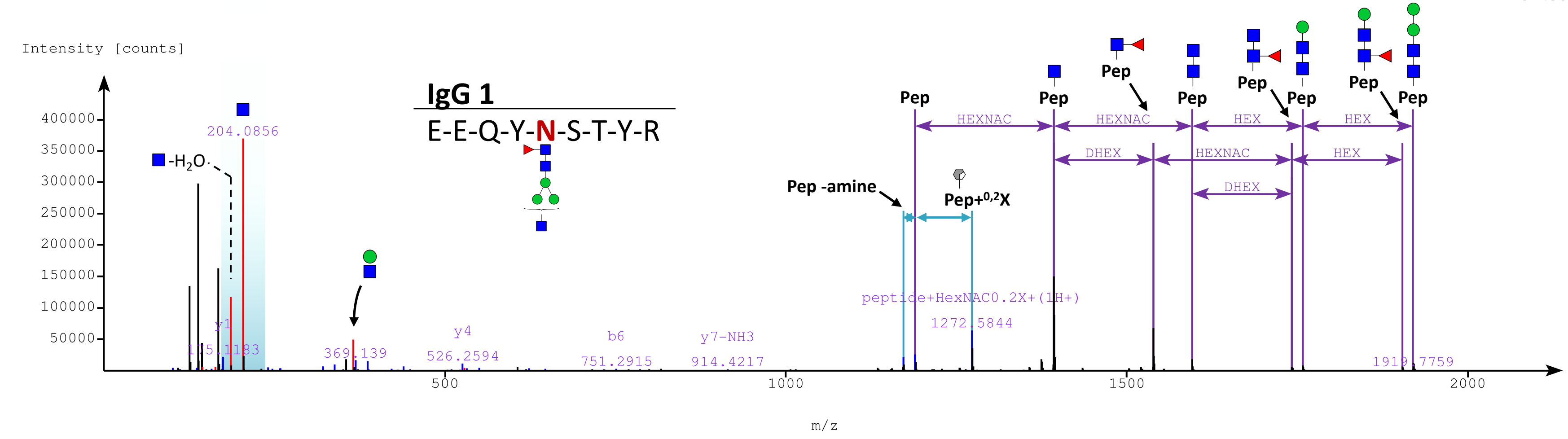
HCD.low

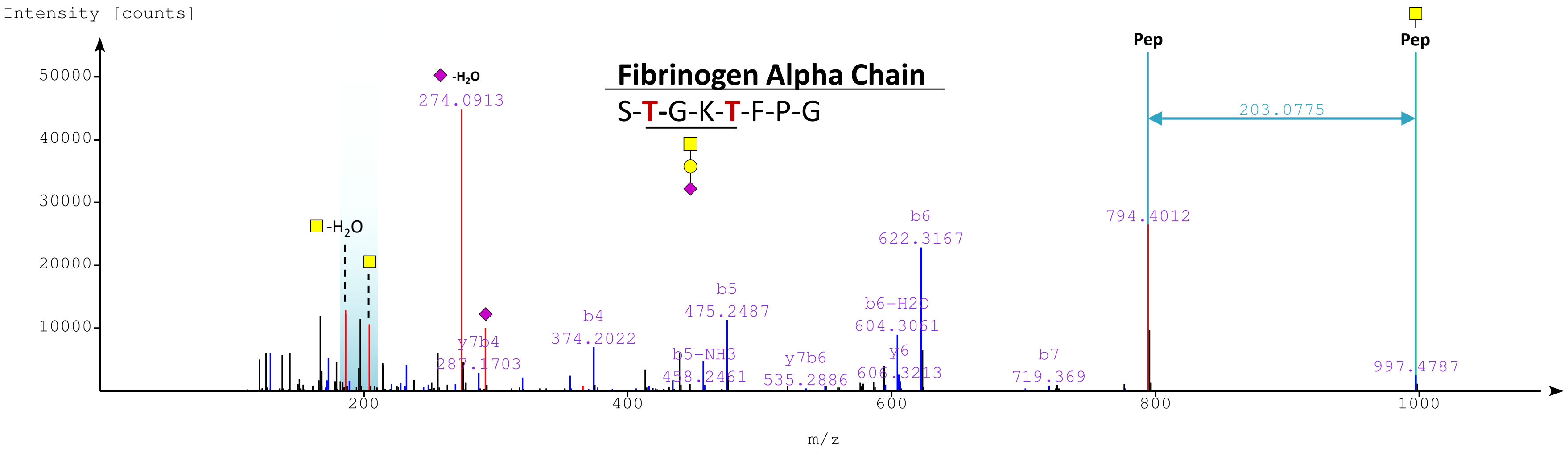
- Hex$_2$ ●● only present with high-mannose- and hybrid-type *N*-glycopeptides

Oxonium Ions: *N*- vs *O*-Glycopeptides

Differentiation based on Oxonium Ion Pattern

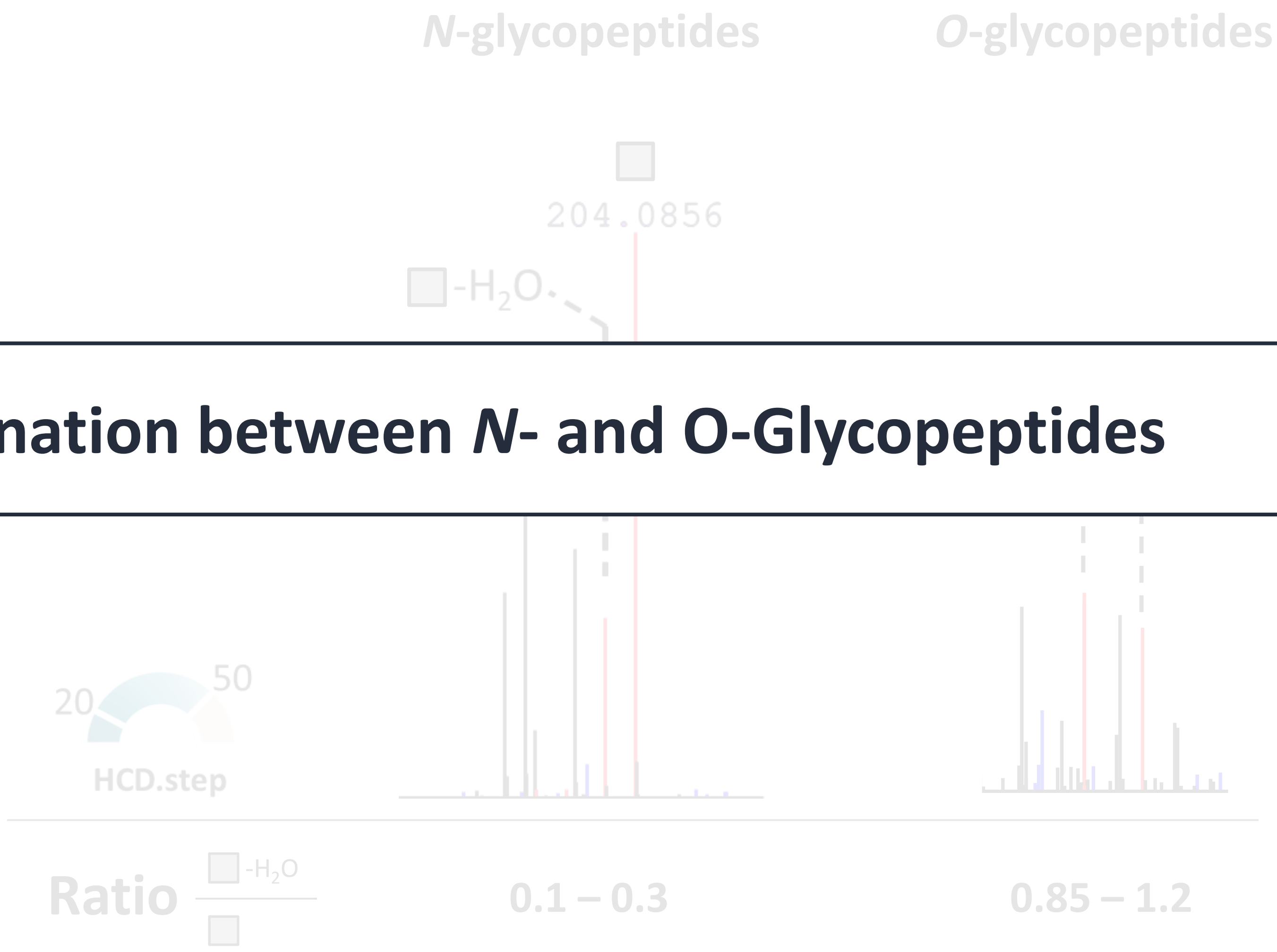
20 50
HCD.step





Oxonium Ions: *N*- vs *O*-Glycopeptides

Differentiation based on Oxonium Ion Pattern



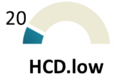
✓

Discrimination between *N*- and O-Glycopeptides

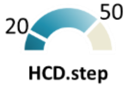
20 50 HCD.step

Summary

N- and *O*-glycoproteomic workflow



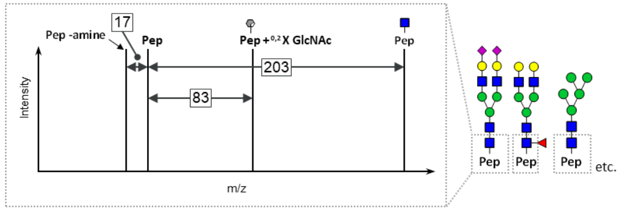
Glycan-derived fragment ions

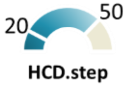


Peptide-derived fragment ions

4 Exemplary Glycoproteins

Conserved fragmentation pattern

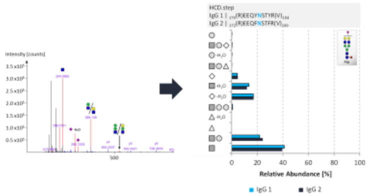




Conserved *N*-glycopeptide fragmentation pattern

Oxonium ion pattern

Systematic evaluation of the diagnostic potential



- Conserved fragmentation pattern: energy-dependent, glycoform-dependent
- Discrimination of complex-, high-mannose- and hybrid-type *N*-glycopeptides; *N*- and *O*-glycopeptides
- Discrimination of glycan features: e.g. antenna vs bisecting GlcNAc, antenna vs core fucosylation, NeuAc vs NeuGc

N- and *O*-glycoproteomic workflow

glyXtoolMS

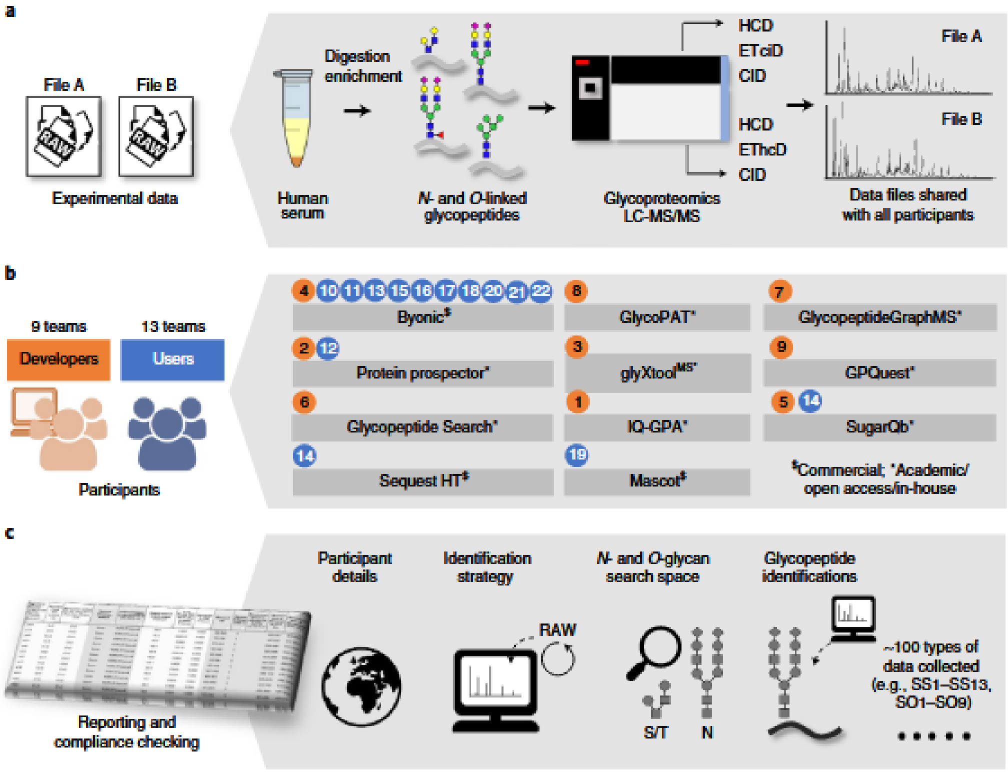
Co-developed with glyXera, open access, open source

[1] Hoffmann *et al. Proteomics*, (2018). [2] Pioch *et al.*, *Anal Chem*, (2018). & Kawahara *et al.*, *Nature Methods, (2021)*

ANALYSIS
https://doi.org/10.1038/s41592-021-01309-x
nature methods

OPEN

Community evaluation of glycoproteomics informatics solutions reveals high-performance search strategies for serum glycopeptide analysis



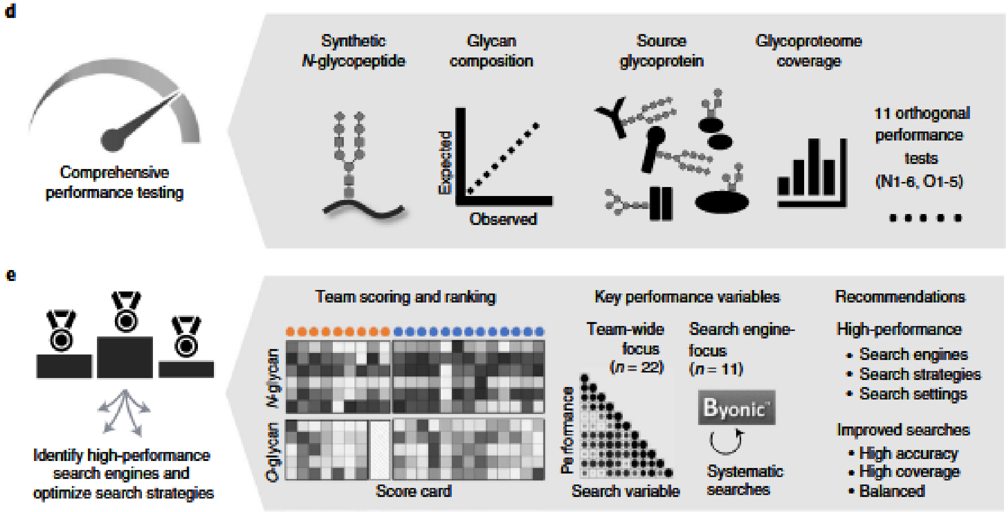
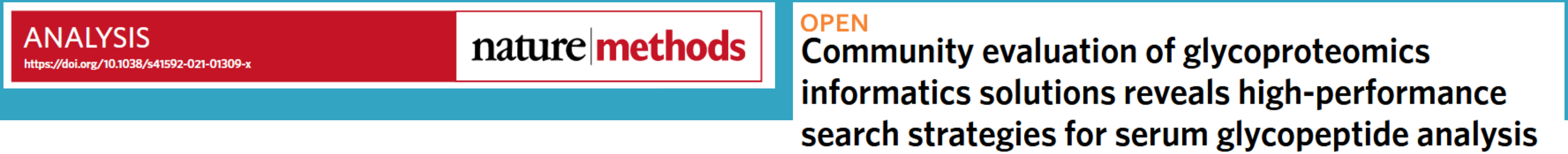


Fig. 1 | Study overview. a, Two glycoproteomics data files of human serum (Files A and B) were generated and shared with participants. **b**, Participants comprising both developers (orange) and users (blue, team identifiers indicated) employed diverse search engines to complete the study. **c**, Teams returned a common reporting template capturing details of the applied search strategy including key search settings (SS1–SS13) and search output (SO1–SO9, Table 1) and their identified glycopeptides. **d**, Complementary performance tests (N1–N6, O1–O5; Table 2) were used to comprehensively evaluate the ability of teams to identify *N*- and *O*-glycopeptides. **e**, The performance profiles were used to score and rank the developers and users separately. Diverse team-wide and search engine-centric (Byonic-focused) approaches were employed to identify performance-associated variables and high-performance search strategies.


ANALYSIS

https://doi.org/10.1038/s41592-021-01309-x

nature methods

OPEN

Community evaluation of glycoproteomics informatics solutions reveals high-performance search strategies for serum glycopeptide analysis

...glyXtoolMS... identified as high-performance N- and O-glycoproteomics software, respectively.

"Overall, Protein Prospector (team 2, overall score 0.682), Byonic (team 4, 0.676) and GlycoPAT (team 8, 0.647) were found to be high-performance software solutions for **N-glycoproteomics**. Notably, our scoring did not separate these three developers by any substantial margin, but their overall performance was slightly higher than that of IQ-GPA (team 1, 0.621) and **glyXtoolMS** (team 3, **0.580**) and substantially higher than that of the other software (score range 0.296–0.366)"

⇨ 5th Place !!!

1st Place !!! ⇦

„Conversely, IQ-GPA (team 1) and **glyXtoolMS** (team 3) were **the best performing software in tests scoring the O-glycan compositions** (O1) **and O-glycoproteome coverage** (O3), respectively. Overall, Protein Prospector (team 2, overall score 0.613) and **glyXtoolMS** (team 3, **0.522**) were found to be high-performance software for O-glycoproteomics"

Summary

N- and *O*-glycoproteomic workflow

Workflow

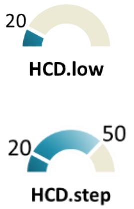
Standard Glycoproteins
Human: Immunoglobulin gamma, Fibrinogen, Lactotransferrin
Bovine: Ribonuclease B

FASP Digest 10 kDa MWCO | **Proteinase K Trypsin**

Glycopeptide Enrichment | **Spin-Cotton-HILIC-SPE**

nanoRP-LC-ESI-OT-OT MS/MS | HCD.low HCD.step Normalized Collisional Energy (NCE)

Data Processing/ Manual Validation | **glyXtool^{MS}**

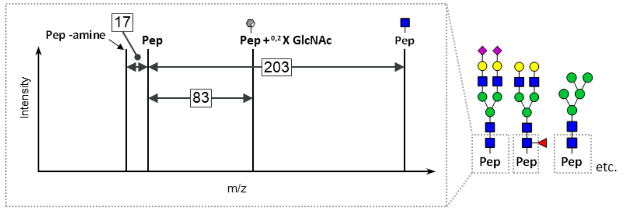


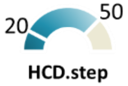
Glycan-derived fragment ions

Peptide-derived fragment ions

4 Exemplary Glycoproteins

Conserved fragmentation pattern

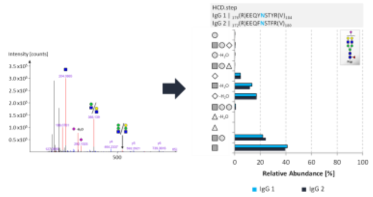




Conserved *N*-glycopeptide fragmentation pattern

Oxonium ion pattern

Systematic evaluation of the diagnostic potential



- Conserved fragmentation pattern: energy-dependent, glycoform-dependent
- Discrimination of complex-, high-mannose- and hybrid-type *N*-glycopeptides; *N*- and *O*-glycopeptides
- Discrimination of glycan features: e.g. antenna vs bisecting GlcNAc, antenna vs core fucosylation, NeuAc vs NeuGc

+

N- and *O*-glycoproteomic workflow

glyXtoolMS

Co-developed with glyXera, open access, open source

In-Depth *N*- and *O*-Glycoproteomics

- Increased Confidence
- Increased Depth of Analysis
- Reduced Analysis Time

[1] Hoffmann *et al. Proteomics*, (2018). [2] Pioch *et al.*, *Anal Chem*, (2018). & Kawahara *et al., Nature Methods, (2021)*

Acknowledgement

- Prof. Udo Reichl
- Dr. Marcus Hoffmann
- Markus Pioch
- Alexander Pralow

and: glyXera GmbH

Funding:

The Concert of Dolichol-Based Glycosylation:
From Molecules to Disease Models

DFG

Research Unit FOR2509

for2509.de