Identification and separation of N-glycans by HILIC/MS

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OVERVIEW
Here we present and compare various approaches for N-glycan analysis of antibodies by evaluating sample preparation, labeling, chromatographic separation, and response using fluorescence detection and electrospray tandem mass spectrometry.

INTRODUCTION
N-glycosylation is a common post-translational modification in proteins that can affect biological function. Therefore glycosylation profiling is important in the characterization of therapeutic proteins such as IgG monoclonal antibodies and fusion proteins. Since there is no single analytical methodology that can achieve full glycan characterization in terms of glycan composition, quantitation, structures and linkages, different analytical approaches need to be utilized.

METHODS
Glycan-based
PNGase F digestion:
• in-solution deglycosylation
• immobilized protein deglycosylation (PA columns)
Labeling of free glycans (vendor protocols):
• 2-Aminobenzamide (2-AB) (Prozyme, Ludger).
• InstantAB (Prozyme)
• Rapiflour-MS (Waters)
Clean-up:
• Cold ethanol precipitation of protein
• HILIC clean-up column (Prozyme)
• HILIC SPE plates (Waters)
Separation and identification:
• HILIC HPLC or UPLC
• ESI TOF MS (details on individual figures)
• Intact mass measurements aided with elution times and/or GU values
Peptide-based
• Immobilized trypsin digestion (Perfinity Flash Digest)
• HILIC separation of glycopeptides (no labeling needed)
• ESI MS

Peptide-based N-glycan analysis

The various glycoforms of the glycopeptide EEQYNSTYR were separated and detected (Thermo XL Orbitrap). Results are compared to labeled N-glycan preparations from the same antibodies analyzed by HILIC/fluorescence/ESI-TOF MS (Agilent 6230 TOP).

Example figures: High mannose glycan series:
The various glycoforms of the glycopeptide EEQYNSTYR were separated and detected (Thermo XL Orbitrap).

Separation profiles of labeled N-glycans

2-Aminobenzamide label:
• Separation is based on hydrophilic interactions and hydrogen bonding
• Robust separation (similar peak capacity and resolution at all conditions)
• Retention times are not dependent on ionic strength (except sialic acid containing glycans)
• InstantAB label:
• Separation is based on both hydrophilic and electrostatic interactions
• Retention and resolution improves at higher ionic strength

Glycan profiling using Rapiflour MS

Sensitivity (mass and relative% of glycans present in trace amounts (~0.5%) can be reproducibly determined)
• Accurate mass coupled to elution order (GU units or relative retention times) aids identification
• Quantitation based on fluorescent – sensitivity, linearity
• Very useful for analyzing complex, late eluting or sialic acid containing glycans

Labeling efficiencies

Percent of unlabeled G0F to total G0F (InstantAB-launched + unlabeled): ~5% (based on EIC areas) – unlabeled may not be present
Percent of unlabeled 2-AB to total 2-AB (RapiflourMS-launched + unlabeled): ~5% (based on EIC areas) – unlabeled may not be present
Percent of unlabeled G1F-GlcNac to total G1F-GlcNac (RapiflourMS-launched + unlabeled): ~7% (based on EIC areas) – unlabeled may not be present