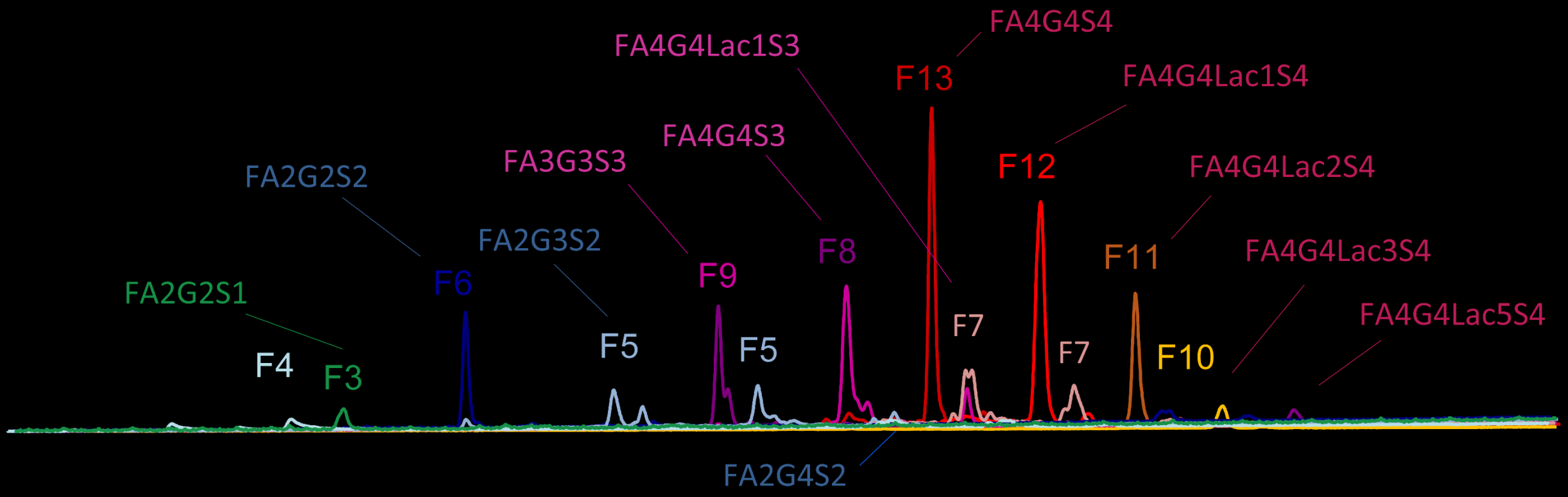
Glycan Analysis Services:

Intact Released Glycan Analysis

Ludger



Glycan Analysis *and why it is important*

Protein glycosylation can play an important role in bioactivity, stability, biological half-life and immunogenicity of a biopharmaceutical. Glycosylation is a post-translational modification and, unlike transcription, is a non-template-driven enzymatic modification process, so glycosylation can change with alterations to production conditions.

Characterisation of glycosylation is required to understand structure-activity relationships. For example, removal of the core fucose from the N-glycan on the heavy chain of IgG_1 can significantly enhance the FcγRIIIa binding affinity, resulting in increased antibody-dependent cellular cytotoxicity activity. Many biopharmaceuticals are produced in nonhuman cell lines which have the potential to add nonhuman-type glycosylation (e.g., Gal α1–3Gal *or* Neu5Gc). This may give rise to altered immunogenicity that can lead to clinical consequences such as reduced efficacy, altered pharmacokinetics, general immune and hypersensitivity reactions, as well as neutralisation of the drug. It is therefore important to identify the Glycosylation Critical Quality Attributes (GCQAs) from a combination of glycan structure analysis, functional tests, clinical data and prior knowledge. Understanding of GCQAs will lead to increased product efficacy and decreased risk of adverse reactions.

Monitoring glycosylation during clone selection, optimisation of culture conditions and purification will also speed up development of the best product and thereby reduce costs.

Quality Control monitoring of glycosylation is required to ensure batch to batch consistency, as well as for comparability studies following any major process changes. Appropriate methods should be chosen for monitoring the drug specific GCQAs.

Due to the importance of glycosylation on drug efficacy and safety, regulatory guidelines require analysis of glycans (e.g. ICH guideline Q6B:Part6.11(f)) for IND submissions.

Analysis of Intact Released Glycans: *Overview*

1. Methods for Releasing Glycans

- What sort of glycans are present? *N-glycans, O-glycans, GSLs?*
- Which release method should be used?

2. Analytical Methods

- What analytical options are there?
- What (if any) label is the best for the specific detection method?
- What level of information is required?

  For characterisation, detailed structural information is required.

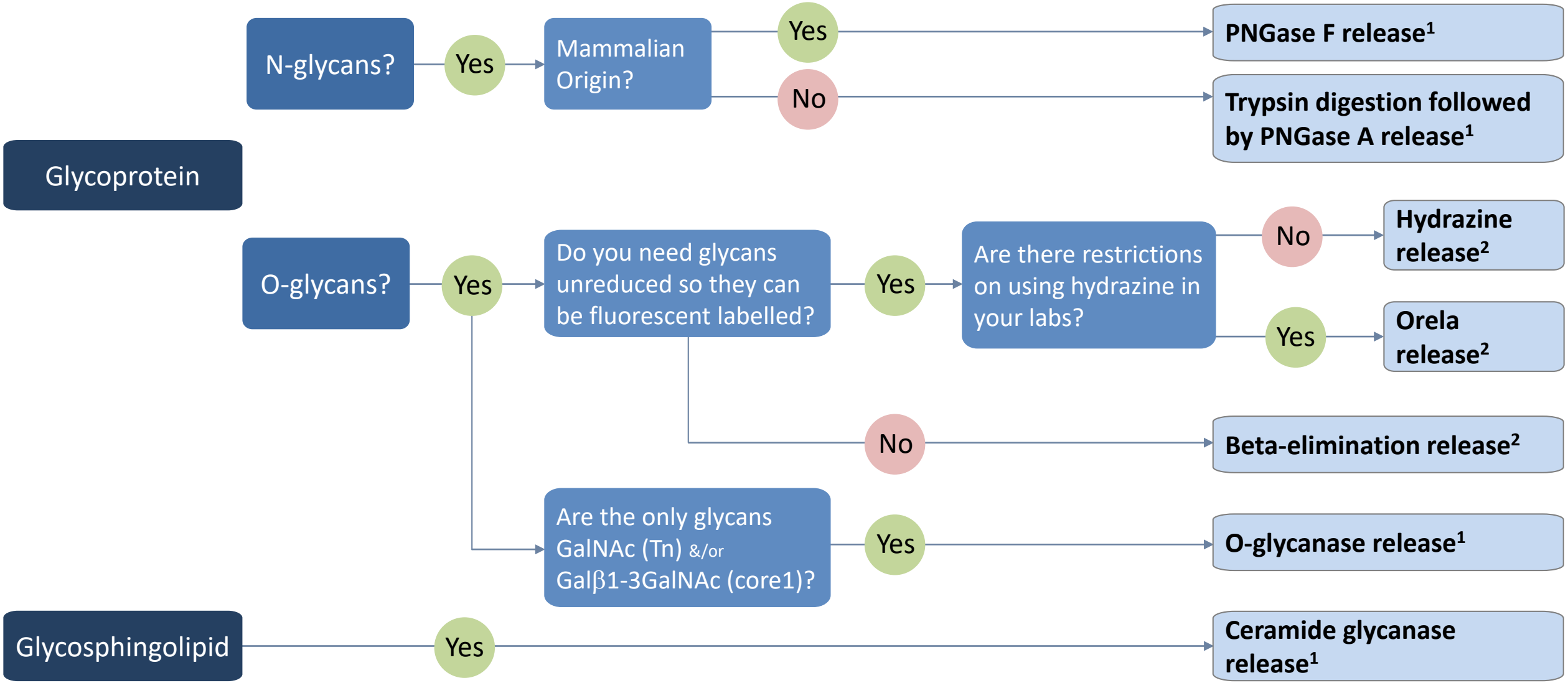
  For QC, profile comparisons to monitor known structures are often sufficient.

3. Examples of Ludger Workflows for Detailed Glycan Characterisation of:

- IgG N-glycans
- EPO N-glycans

4. Examples of QC Analysis Methods for specific GCQAs

Releasing Glycans: *How to choose a method*



Note: [1]Enzymatic release is more specific than chemical release, and chemical release[2] can lead to some degradation of the glycan (i.e. peeling; Ref: Kozak R. et al Anal Bioch 423, 2012,119-128)

Glycan Analytical Methods *and What Information is Obtained*

HILIC-LC Separation

Separation by size (and shape)

- Glucose units (GU, which are standardised values of retention times compared to a glucose homopolymer ladder) that can be matched to standards and to database values for preliminary identification. Retention time is roughly proportional to size, with some additional separation of isomers due to shape
- Relative proportions from peak areas

WEAK Anion Exchange (WAX) Charge Separation

Separation by charge (and size)

- Retention times can be matched to standards for identification of mono-, di-, tri- and tetra-sialylated glycans.
- Where two structures have the same charge, the smallest structure (with the least sugars) will elute later
- Phosphorylated and sulphated glycans will also be retained
- Relative proportions from peak areas

MS, Mass separation

Separation by mass

- Mass composition data gives numbers of monosaccharide units:
  - *Hexose (H)*
  - *N-acetylhexosamine (N)*
  - *deoxyhexosamine (usually fucose: F)*
  - *Phosphate or Sulphate – Note: mass difference is only 0.009 mass units so need complementary data to distinguish between them.*
  - *Sialic acids: Neu5Ac (S); Neu5Gc (Sg); O-acetylated sialic acid (+Ac) - Note: sialic acids are unstable during MALDI analysis. Permethylation can be used to stabilise them.*

Exoglycosidase Sequencing (Exo) & HILIC-LC

Digestion with exoglycosidases specific for monosaccharide and linkage

- Peaks shift following removal of specific monosaccharides and linkages enable identification of specific epitopes
  e.g. Core alpha 1-6 fucose, alpha linked galactose
- Shift in GU or m/z provides data on number of monosaccharides removed *e.g. 1 or 2 Gal*
- Relative proportions of different epitopes from peak areas

Exoglycosidase Sequencing & WAX-LC

Exoglycosidases specific for charged structures

- Sialic acid peaks shift following sialidase digestion
- Phosphorylated peaks shift following phosphatase digestion

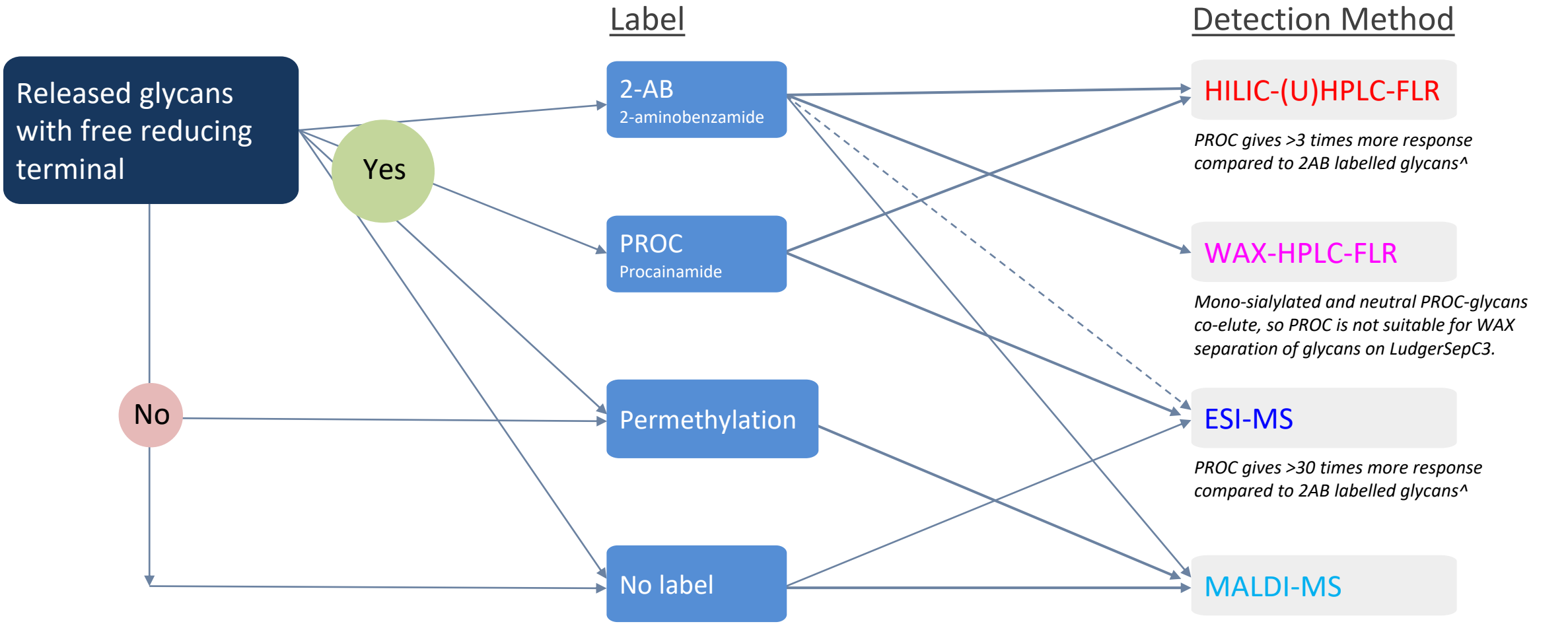
MS/MS Fragmentation

Fragmentation followed by separation by mass

- Mass composition sequence data

HILIC = hydrophilic interaction chromatography; (U)HPLC = (ultra) high performance liquid chromatography; FLR = fluorescence detection; WAX = weak anion exchange; Exo = exoglycosidase treatment; MS = mass spectrometry; MALDI = Matrix Assisted Laser Desorption/Ionisation; ESI = electrospray ionisation; MS/MS = mass fragmentation.

Selecting a Glycan Label for the Analytical Detection Method



Labelling with 2AB or PROC is by reductive amination to the hydroxyl group on the reducing terminal of sugars.
Note that other commercially available 'instant' labelling systems labels label the amino group formed during PNGase F release before hydrolysis of the reducing terminal of the sugar. Thus, these systems cannot be used to label any pre-released N-glycan standards or other sugars with free reducing ends such as O-glycans, GSL-glycans or GAGs.

^ Reference: Kozak RP, et al. Anal Biochem 2015, 486:38-40

What Level of Information is Required?

Detailed Characterisation

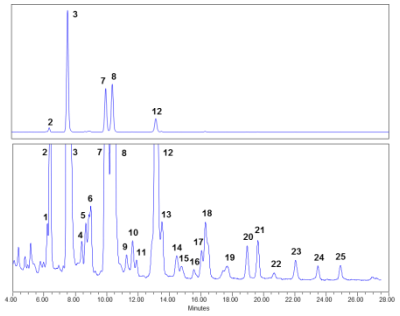
- During drug development it is important to know which glycans are present and their relative proportions. Thus detailed glycan characterisation is required. This data can then be used in combination with functional tests, clinical data and prior knowledge to determine the Glycosylation Critical Quality Attributes (GCQAs).
- A number of analytical methods can be combined -cross correlation of complementary data provides increased confidence in structure assignments.
- Examples of Ludger workflows for detailed characterisation of N-glycan from IgG and EPO are shown in the following slides.

Comparison Profiling

- Quality Control monitoring of glycosylation is required to ensure batch to batch consistency, as well as for comparability studies following any major process changes.
- For QC, profile comparisons to monitor known structures (e.g GCQAs) are often sufficient.
- The most appropriate analytical method(s) can be adopted once detailed characterisation has been performed
- Examples of QC analysis methods for specific GCQAs are shown in the following slides

Detailed Characterisation Workflow for IgG N-Glycans

HILIC-(U)HPLC-FLR

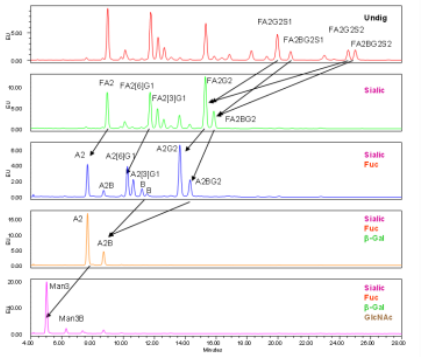


HILIC-UHPLC profile for comparability studies or QC

Peak id	Average		%CV
	GU	% Area	
1	5.42	0.24	4.72
2	5.50	1.59	0.49
3	5.97	45.41	0.74
4	6.28	0.28	19.13
5	6.37	0.34	0.25
6	6.48	0.75	1.74
7	6.80	18.61	0.30
8	6.92	21.51	0.56
9	7.18	0.19	2.01
10	7.30	0.28	2.06
11	7.37	0.11	2.37
12	7.73	7.33	2.25
13	7.83	0.46	5.89
14	8.10	0.24	8.52

Glucose unit (GU) values for preliminary structure assignments.
Relative proportions (%Area) of glycan peaks

HILIC-(U)HPLC-FLR-Exo



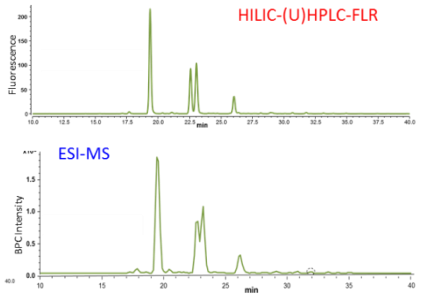
HILIC-UHPLC profiles with GU before and after specific exoglycosidases for comparability studies or QC

Type of Structure	Total %
Sialylated	32.4
High Mannose	2.7
Fucosylated	82.2
Bianatennary	98.7
Tri antennary	0.35
G0	19.6
G1	32.9
G2	43.5

Structure Assignments with monosaccharide type, order and linkage information
Relative proportions (% Area) of glycan peaks e.g.

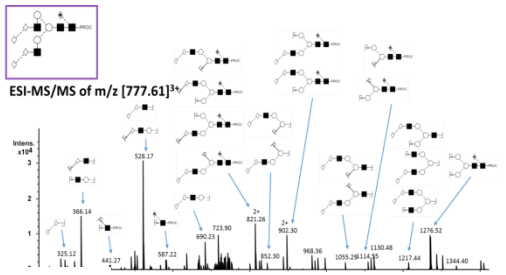
G0:G1:G2
A1:A2:A3
% fucosylation
% sialylation
% high mannose
% Galα1-3Gal

HILIC-(U)HPLC-FLR-ESI-MS/MS



HILIC-UHPLC profile
GU values & % Area

MS/MS Sequencing



Mass composition sequence data
Matching of GU, m/z & MS/MS sequencing data.

G0:G1:G2
A1:A2:A3
fucosylation
sialylation
high mannose

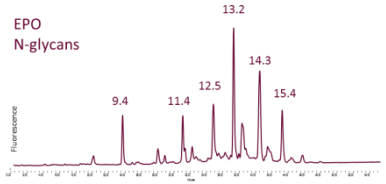
Cross -correlation of data from GU, m/z, MS/MS & exoglycosidase sequencing

Structure	HILIC-UPLC		ESI-MS/MS						Degree of Certainty			
			Composition									
	GU	% Area	Hex (H)	HexNAc (N)	Fucose (F)	NeuGc (Sg)	Mass found	Mass Calculated	GU	Exoglycosidase digestions	m/z at GU	MS/MS at GU
FA2[3]G1Ga1	7.46	0.56	5	4	1	0	[669.61]$^{2+}$	[669.62]$^{2+}$	Y	Y	Y	Y
FA3G2	7.61	0.18	5	5	1	0	[732.22]$^{2+}$	[731.31]$^{2+}$	Y	Y	Y	Y
FA3G2	7.69	0.26	5	5	1	0	[737.31]$^{2+}$	[737.32]$^{2+}$	Y	Y	Y	Y
FA3G1Ga1	7.72	0.21	5	5	1	0	[737.31]$^{2+}$	[737.32]$^{2+}$	Y	Y	Y	Y
FA2[6]G1Sg1	7.97	0.10	4	3	1	0	[717.94]$^{2+}$	[717.96]$^{2+}$	Y	Y	Y	Y
FA2[3]G1Sg1	8.10	0.34	5	4	1	1	[771.98]$^{2+}$	[771.99]$^{2+}$	Y	Y	Y	Y
FA2G2[6]Ga1	8.22	1.09	6	4	1	0	[723.60]$^{3+}$	[723.63]$^{3+}$	Y	Y	Y	Y
FA2G2[3]Ga1	8.45	0.10	6	4	1	0	[723.60]$^{3+}$	[723.63]$^{3+}$	Y	Y	Y	Y
FA3G3	8.57	0.28	6	5	1	0	[791.28]$^{3+}$	[791.33]$^{3+}$	Y	Y	Y	Y
FA2G2Sg1	8.89	0.50	5	4	1	1	[791.28]$^{3+}$	[791.33]$^{3+}$	Y	Y	Y	Y
FA2G2Ga2	9.12	0.78	7	4	1	0	[771.93]$^{4+}$	[771.98]$^{4+}$	Y	Y	Y	Y
FA2G2Ga1Sg1	9.39	0.16	6	4	1	1	[825.94]$^{3+}$	[826.00]$^{3+}$	Y	Y	Y	Y
FA3G2Ga2 and FA3G3Ga1			7	4	1	0	[845.64]$^{3+}$	[845.33]$^{3+}$	Y	Y	Y	Y
FA2G2Ga1Sg1	9.73	0.43	6	4	1	0	[825.95]$^{3+}$	[826.00]$^{3+}$	Y	Y	Y	Y
A2G2Sg2	10.11	0.26	5	4	0	2	[825.62]$^{3+}$	[826.00]$^{3+}$	Y	Y	Y	Y
FA2G2Sg2	10.50	0.32	5	4	0	2	[874.29]$^{3+}$	[874.68]$^{3+}$	Y	Y	Y	Y
FA3G3Ga3	11.17	0.14	9	5	1	0	[953.38]$^{3+}$	[953.30]$^{3+}$	Y	Y	Y	Y

Increased confidence in assignments

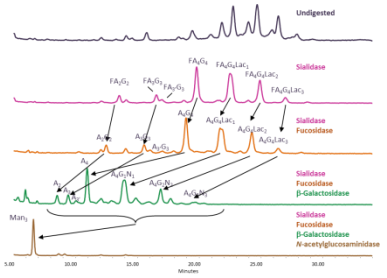
Detailed Characterisation Workflow for EPO N-Glycans

HILIC-(U)HPLC-FLR



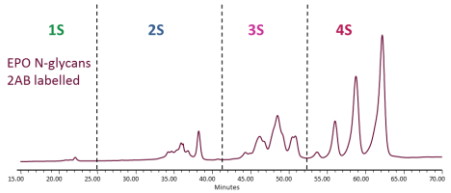
- HILIC-UHPLC profile for comparability studies or QC
- GU values for preliminary structure assignments.
- % Area of glycan peaks

HILIC-(U)HPLC-FLR-Exo



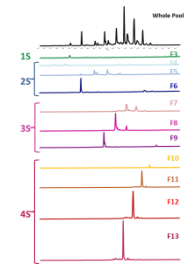
- Structure assignments with monosaccharide order and linkage information
- % Area of glycan peaks
- % Antennary Structures
- % Lactosamines repeats
- O-acetylated sialic acids.

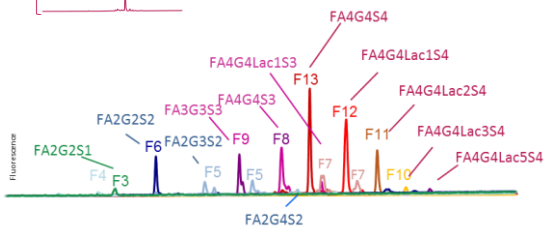
WAX-HPLC-FLR



- Charge profile for comparability studies or QC
- % Area of glycans with 1, 2, 3 & 4 sialic acids

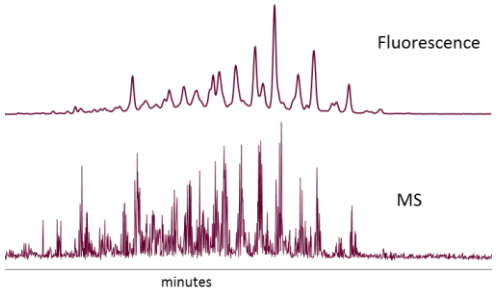
WAX-HPLC-FLR-HILIC(U)HPLC-FLR-Exo





- Identification of N-glycan structures with 0,1,2,3 or 4 sialic acids-
- monosaccharide type, linkage and sequence

HILIC-(U)HPLC-FLR-ESI-MS/MS



GU value	% Area	% CV	Composition						
			Hex (H)	HexNAc (N)	Fuc (F)	Neu5Ac (S)			potential sulphate or phosphate
						0 OAc	1 OAc	2 OAc	
6.63	0.02	67.10	5	4	1	1	0	1	0
6.78, 6.88	0.22	16.20	5	2	0	0	0	0	1
7.90	0.24	10.75	5	4	1	1	0	1	0

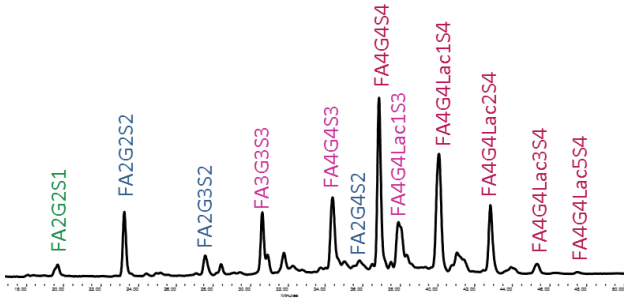
- HILIC-UHPLC profile with glucose unit (GU) values and % Areas
- m/z profile with mass composition matched to LC peaks
- Mass composition sequence data
- Matching of GU, m/z & MS/MS for identification of structures

Cross-correlation of data from GU, charge, m/z, MS/MS & exoglycosidase sequencing

GU value	% Area	Composition						
		Structure	Hex (H)	HexNAc (N)	Fuc (F)	Neu5Ac (S)		
						0 OAc	1 OAc	2 OAc
10.00	6.19	FA3G3S3	6	5	1	3	0	0
10.50	10.12	FA4G4S4Ac1	7	6	1	3	1	0
10.95	7.88	FA4G4S3	7	6	1	3	0	0
11.41	13.46	FA4G4S4	7	6	1	4	0	0
12.41	7.15	FA4G4S4(LacNAc)1	8	7	1	4	0	0
13.37	2.98	FA4G4S4(LacNAc)2	9	8	1	4	0	0
14.31	0.52	FA4G4S4(LacNac)3	10	9	1	4	0	0

- Assignments made from cross-correlation of complementary analyses.
- The more data, the higher the confidence in the structure assignments

- Individual N-glycan structures: monosaccharides and linkages (as far as possible)
- Relative % of glycans carrying specific epitopes
- Relative % of antennary and poly-lactosamine structures
- Relative % of sialylated structures (0,1,2,3,4 sialic acids)
- Relative % of fucosylated structures

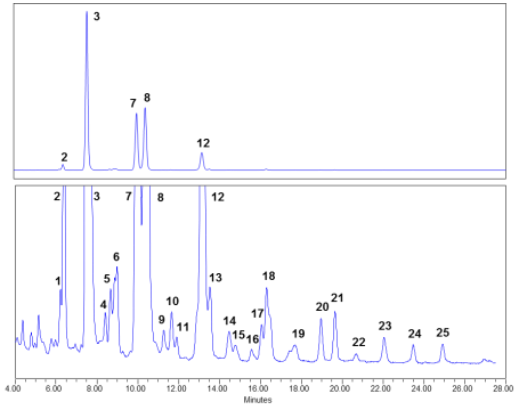


Examples of QC Analysis Methods for IgG GCQAs

G0:G1:G2; Core Fucose; Sialic acids; High Mannose

- Galactosylation, core fucosylation, sialylation and high mannose structures are all GCQAs as they can effect the function of Mabs.
- HILIC -UHPLC with fluorescent detection (HILIC-UHPLC-FLR) provides the relative proportions of these different glycan structures (which have been previously identified by detailed characterisation).

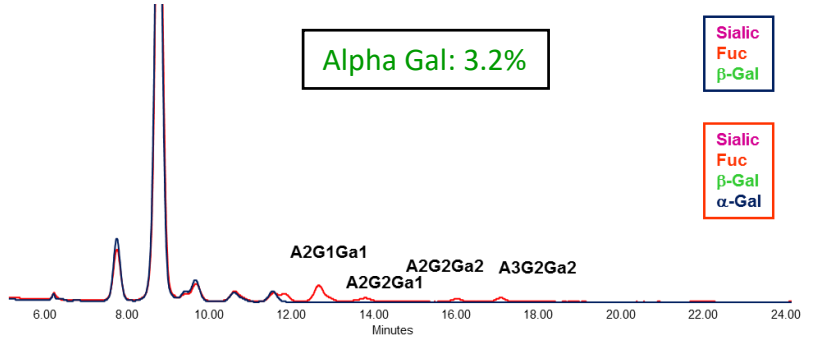
Type of Structure	Total %
Sialylated	32.4
High Mannose	2.7
Fucosylated	82.2
Bianatennary	98.7
Tri antennary	0.35
G0	19.6
G1	32.9
G2	43.5



Method: HILIC-(U)HPLC-FLR

Gal α1-3Gal

- The epitope Galα1-3Gal is potentially immunogenic. However, structures containing β or α galactose can be difficult to separate by HILIC and have the same mass composition so do not separate by MS.
- HILIC (U)HPLC with fluorescent detection *plus* exoglycosidase treatment (HILIC-(U)HPLC-FLR-Exo) is required to detect the presence of glycans containing the epitope Galα1-3Gal.
- Comparison of profiles following digestion with sialidase, fucosidase and β-galactosidase (+/- α-galactosidase) allow for the detection and relative quantitation of structures containing this epitope.

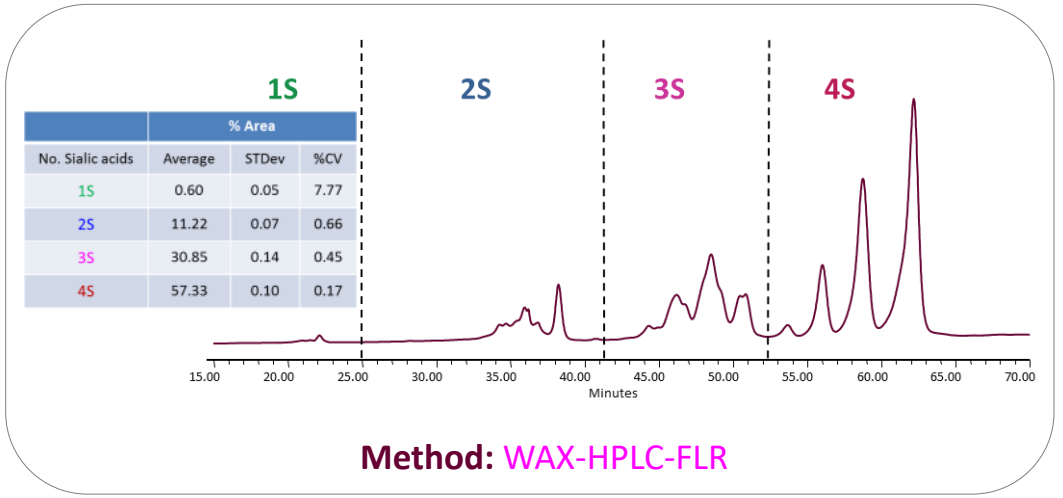


Method: HILIC-(U)HPLC-FLR-Exo

Examples of QC Analysis Methods for EPO GCQAs

Degree of Sialylation

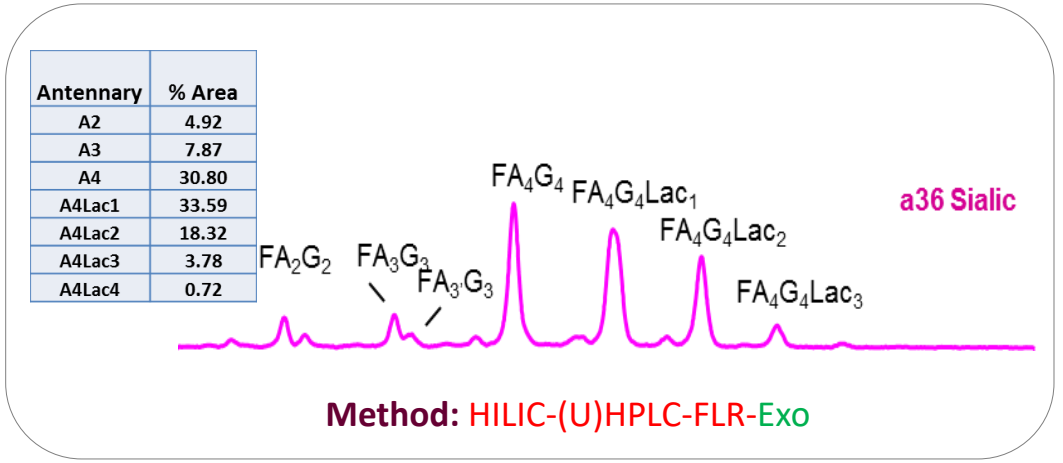
- The degree of sialylation is a GCQA for EPO (and many other glycoproteins) as it can effect the glycoprotein's function and half-life.
- Weak anion exchange HPLC with fluorescent detection (WAX-HPLC-FLR) provides the relative proportions of structures with one or more sialic acid.
- Comparison of individual peaks within the profile can provide information on specific structures where detailed characterisation has been performed to identify which structures are present in each peak.



Method: WAX-HPLC-FLR

Antennary Structures & Lactosamine extensions

- Mono-, bi-, tri- and tetra-antennary structures arise from the addition of the lactosamine sequence Gal-GlcNAc to Man3. These antennary structures can also have Gal-GlcNAc added to them as lactosamine extensions. These isomers can have the same mass composition so do not separate by MS, but can be separated by HILIC.
- HILIC (U)HPLC with fluorescent detection *after* exoglycosidase treatment with sialidase (HILIC-(U)HPLC-FLR-Exo) is required to gain data on the relative proportion of these structures. Due to the large diversity in sialylation of the different glycans, sialidase digestion is required to simplify the profile enough to allow separation of these neutral glycans.



Method: HILIC-(U)HPLC-FLR-Exo