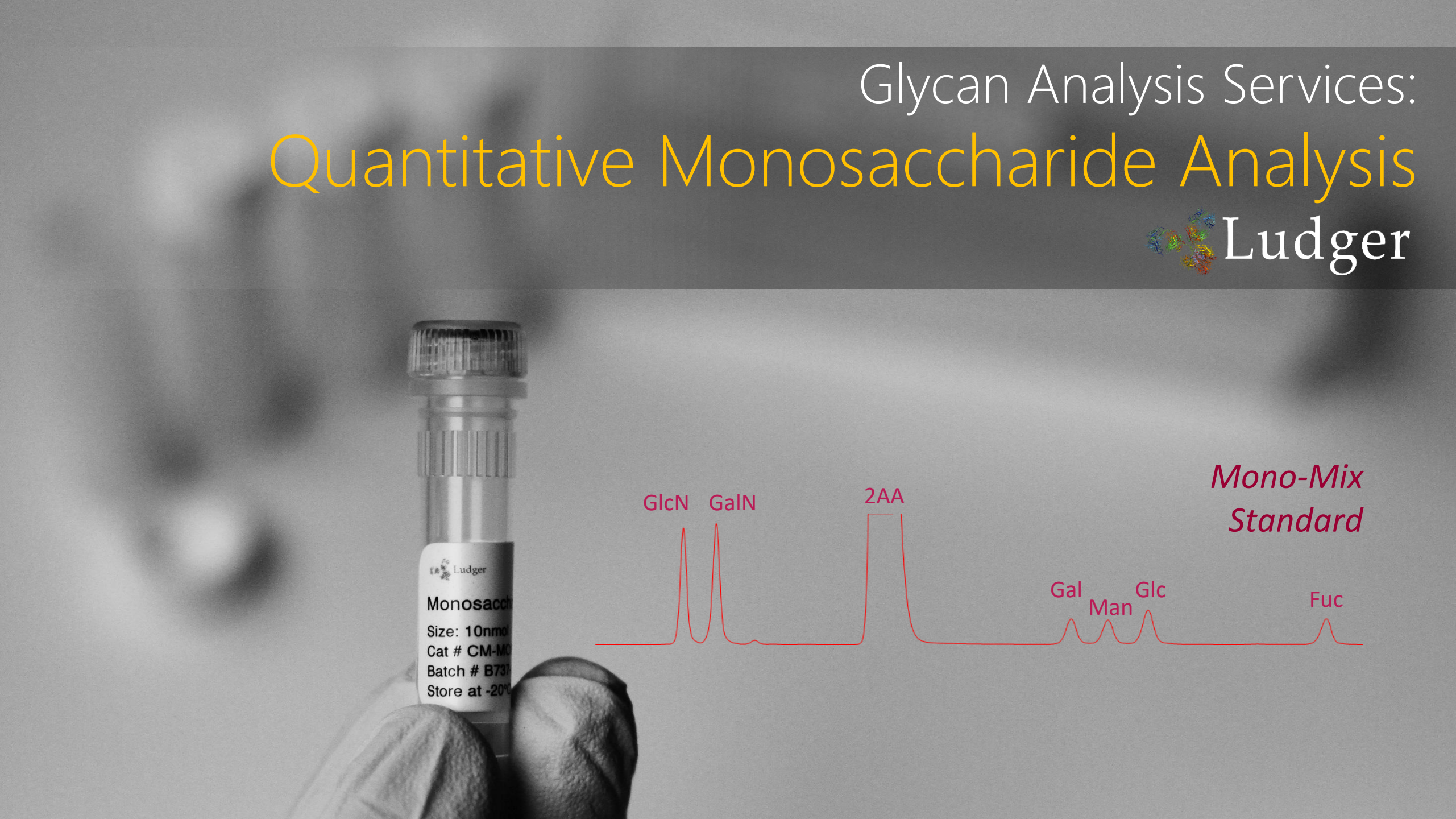
Glycan Analysis Services:

Quantitative Monosaccharide Analysis

Ludger



Quantitative Monosaccharide Analysis *and why it is important*

ICH guideline Q6B states that the carbohydrate content (neutral sugars, amino sugars, and sialic acids) should be determined for glycoprotein biopharmaceuticals. This provides information relating to the types of N- and/or O-glycans present on a glycoprotein. Changes in glycosylation can have a significant affect on both the physiochemical and biological properties of a glycoprotein. It is therefore important to monitor the monosaccharide content during all stages of the product life cycle as well as QC batch to batch consistency.

The major monosaccharides that make up N-glycans and O-glycans are the neutral monosaccharides *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), galactose (Gal), glucose (Glc), mannose (Man) and fucose (Fuc); plus sialic acids. For monosaccharide analysis these sugars are released from the protein by acid hydrolysis. Sialic acids are released under milder conditions than those used to release neutral sugars as sialic acids are destroyed under the conditions required for neutral monosaccharide release, therefore sialic acid quantification is performed separately (view our presentation on Quantitative Sialic Acid Analysis).

Neutral sugars are hydrolysed by incubation with trifluoroacetic acid (TFA) or hydrochloric acid (HCl). Usually 3 hour incubation at 100 °C with 2M TFA will release all of the monosaccharides. If harsher conditions are required (as can be the case to completely remove the core *N*-acetylhexosamines which are directly attached to the protein) 6M HCL can be used, however there will be degradation of the hexose sugars under these conditions, therefore release conditions may require optimising for individual glycoproteins. Also during hydrolysis, *N*-acetyl groups on GlcNAc and GalNAc are hydrolysed to glucosamine (GlcN) and galactosamine (GalN). When quantifying monosaccharides it is important to subject any monosaccharide standards to the same hydrolysis conditions as the glycoproteins as the different sugars degrade at different rates and have different molar fluorescence.

Ludger's Method – using our LT-MONO-96 kit

Neutral monosaccharides are released from the glycoprotein by acid hydrolysis using:
2M trifluoroacetic acid (TFA) *or* 6M hydrochloric acid (HCl)

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Samples are labelled with the fluorescent 2AA tag

↓

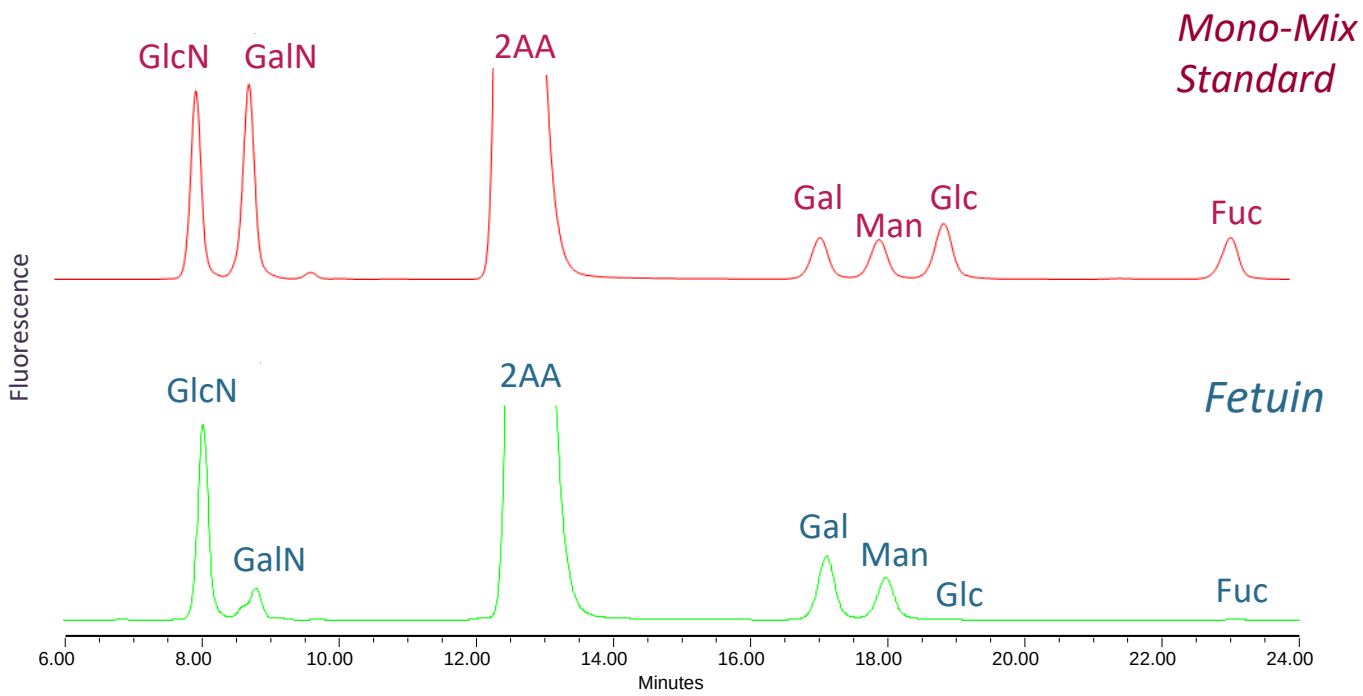
Samples are run on RP-LC

↓

Data analysis.
The absolute amounts of monosaccharides *N*-acetylglucosamine (GlcN); *N*-acetylgalactosamine (GalN); galactose (Gal), mannose (Man), glucose (Glc) and fucose (Fuc) are calculated by reference to standard curves from Mono-Mix quantitative standard.

Quantitative Monosaccharide Analysis: Fetuin TFA Release

- Quantitation of monosaccharides as nmol/mg protein

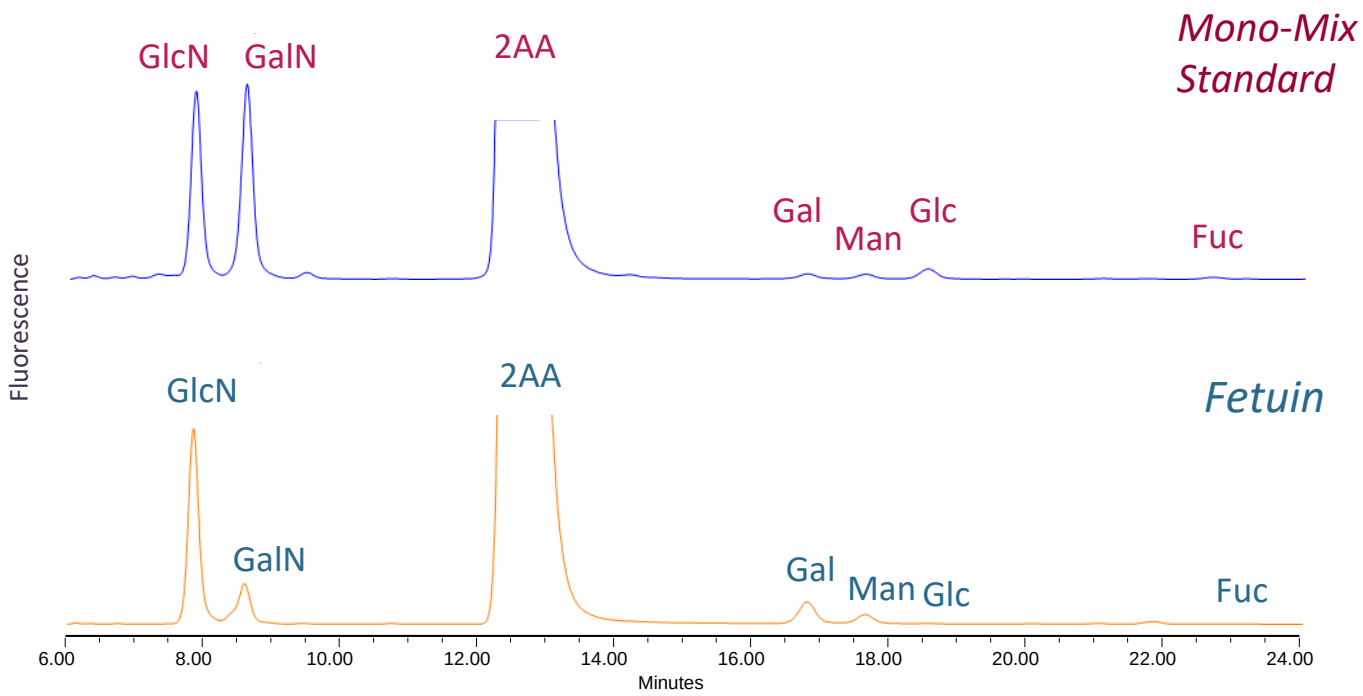


Monosaccharides quantified in nmol/mg protein by reference to standard curves

TFA Release	nmol/mg protein	
Monosaccharide	Average	%CV
GlcN	260	1.3
GalN	50	1.3
Gal	409	6.6
Man	288	5.4
Glc	13	14.5
Fuc	14	2.1

Quantitative Monosaccharide Analysis: Fetuin HCl Release

- Quantitation of monosaccharides as nmol/mg protein



Monosaccharides quantified in nmol/mg protein by reference to standard curves

HCl Release	nmol/mg protein	
Monosaccharide	Average	%CV
GlcN	336	13.1
GalN	70	17.2
Gal*	1071	30.0
Man*	486	27.7
Glc*	8	92.4
Fuc*	0	-

HCl may be required to ensure that the core monosaccharide is fully released (particularly when O-glycans are present).

**A side effect of using the stronger acid is that decomposition of the hexose sugars can occur, so the data is not accurate for the hexose sugars.*

Quantitative Monosaccharide Analysis of Fetuin: Comparison to Published Data

- Comparison of data to published values

HCL & TFA Release Data	nmol/mg protein		Amount Relative to CARC^ Fetuin Analysis
Monosaccharide	Average	%CV	Relative %
GlcN	336	13.1	78
GalN	70	17.2	105
Gal	409	6.6	132
Man	288	5.4	120
Glc	13	14.5	-
Fuc	14	2.1	-

^ http://www.abrf.org/ABRFNews/1997/March1997/mar97monosach.html

The combined data from both releases in in good agreement with published values.

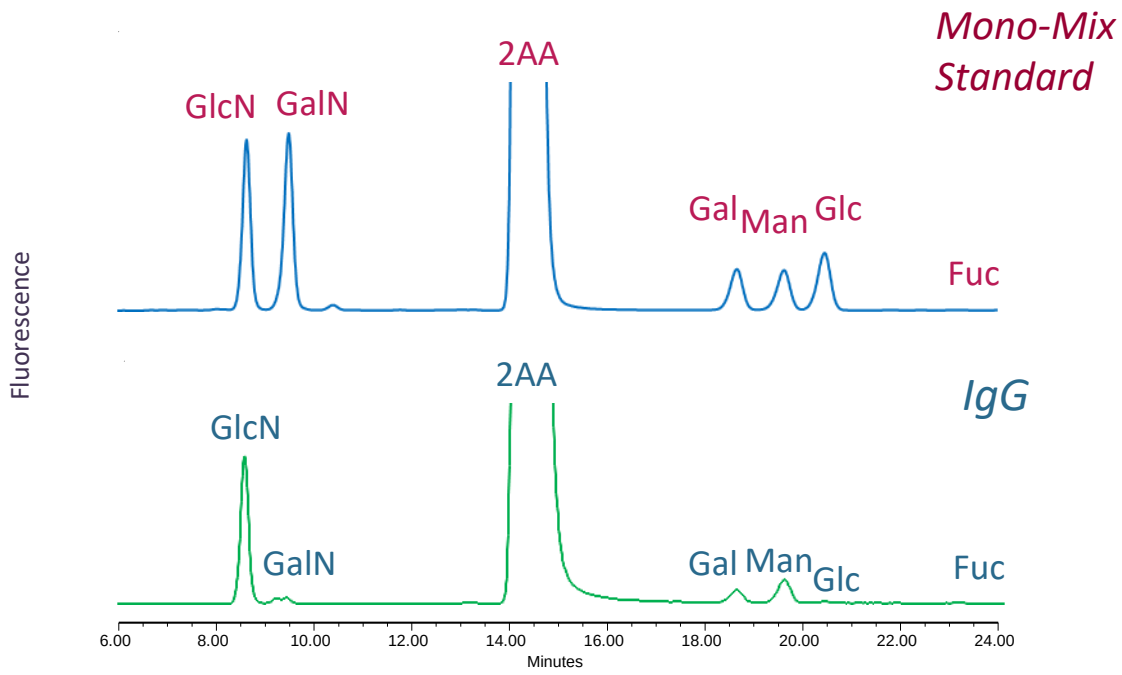
- Data for GlcN and GalN is taken from the HCl release where the stronger acid release more of the core sugars.
- Data for Gal, Man, Glc & Fuc taken from TFA release. Where there is less degradation than with the HCl release.

Fetuin has both N- and O-glycans. On this protein the GalN is from O-glycans.

Glc is present as a background contaminant and is also detected in the negative controls.

Quantitative Monosaccharide Analysis: IgG TFA Release

- Quantitation of monosaccharides as nmol/mg protein

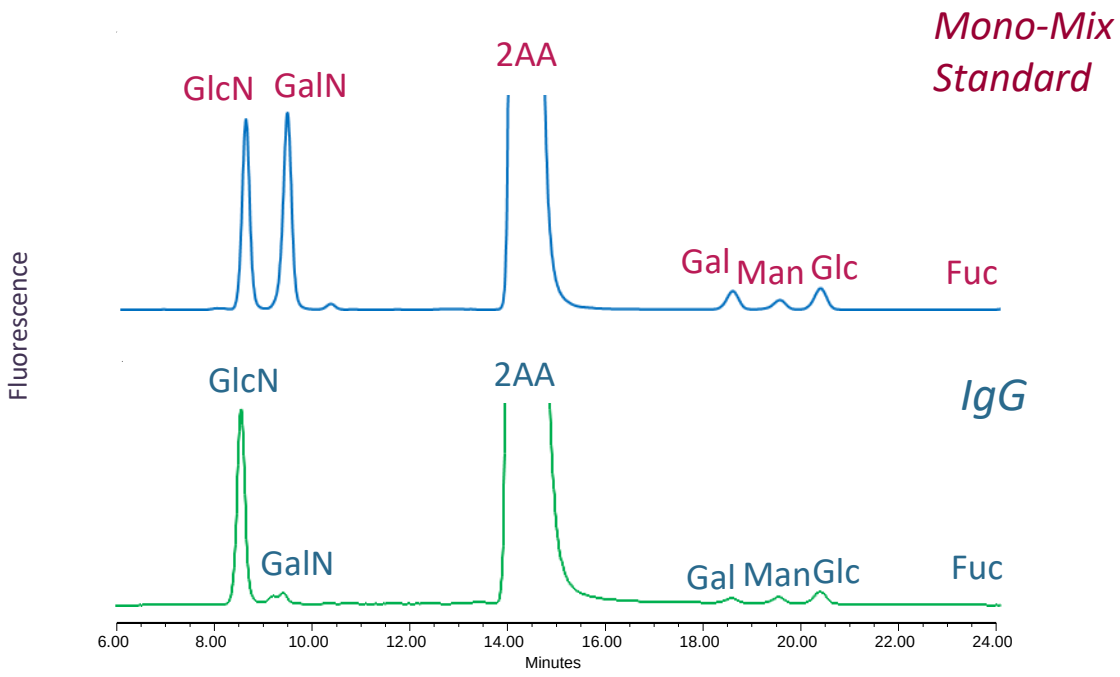


Monosaccharides quantified in nmol/mg protein by reference to standard curves

TFA Release	nmol/mg protein	
Monosaccharide	Average	%CV
GlcN	47	1.7
GalN	8	0.5
Gal	24	2.4
Man	41	1.6
Glc	5	9.2
Fuc	6	1.3

Quantitative Monosaccharide Analysis: IgG HCl Release

- Quantitation of monosaccharides as nmol/mg protein



Monosaccharides quantified in nmol/mg protein by reference to standard curves

HCl Release	nmol/mg protein	
Monosaccharide	Average	%CV
GlcN	58	2.1
GalN	3	2.0
Gal*	28	7.5
Man*	54	1.6
Glc*	19	34.3
Fuc*	20	18.0

HCl may be required to ensure that the core monosaccharide is fully released (particularly when O-glycans are present).

**A side effect of using the stronger acid is that decomposition of the hexose sugars can occur, so the data is not accurate for the hexose sugars.*

Quantitative Monosaccharide Analysis of IgG: Number per N-glycan Site

- Comparison of data to N-glycan Data

HCL & TFA Release Data	nmol/mg protein		Number of monosaccharides per N-glycan site
Monosaccharide	Average	%CV	Number per site
GlcN	58	2.1	4.4
GalN	3	2.0	0.2
Gal	24	2.4	1.8
Man	41	1.6	3.1
Glc	5	9.2	0.4
Fuc	6	1.3	1.2

The combined data from both releases in in good agreement with data from N-glycan analysis.

- Data for GlcN and GalN is taken from the HCl release where the stronger acid releases more of the core sugars.
  - The majority of the N-glycans are biantennary (with 4 GlcN).
  - There are a low percentage of IgGs in human serum that have O-glycans, hence a low, but real GalN value.
- Data for Gal, Man, Glc & Fuc taken from TFA release where there is less degradation than with the HCl release.
  - Not all N-glycans have 2 Gal.
  - All of the complex N-glycans have 3 Man; there is only a very low % of high mannose structures present.
  - The majority of the N-glycans are core fucosylated.
  - Glc is present as a background contaminant and is also detected in the negative controls.