Analytical standards for reliable quantitation of N-glycans in biopharmaceutical characterisation and comparability studies

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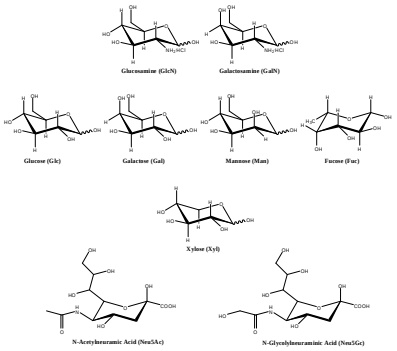
Introduction

Glycosylation features are high priority Critical Quality Attributes (CQAs) for most biopharmaceuticals produced in mammalian expression systems. This is due to the potentially significant impact of the glycans on the safety and efficacy profiles of therapeutic glycoproteins. A difficulty that biopharma companies face is that they have to perform absolute quantitative monosaccharide composition analysis (AQMCA) to demonstrate structural consistency of their glycosylated therapeutics to some regulators, particularly the FDA. However, although it sounds as if it should be simple, obtaining accurate, precise AQMCA data is very challenging. The focus of this work being the development and application of traceable quantitative monosaccharide standards for reliable quantitation of biopharmaceutical glycosylation. The poster overviews the metrology of the orthogonal methods used for quantitation of the BioQuant monosaccharide standards themselves, traceability, and their applications as both internal and external analytical standards for AQMCA work. Finally, we will explain how we use the BioQuant standards in the Ludger Monosaccharide Analysis Kit to obtain reliable monosaccharide composition data in comparability studies of biopharmaceutical N-glycosylation for regulatory submissions.

Results

Monosaccharide standards



A bulk solution of each individual monosaccharide was prepared and the concentration was calculated for each bulk by weight and by qNMR. The agreement between the two techniques is shown in Table 1.

	A: Concentration by weight (mM)	B*: Concentration using by qNMR (mM)	Std dev	CV	B/A Difference
Glucose	0.1998	0.1982	0.004	2.029	99.2
Mannose	0.1998	0.1964	0.004	2.071	98.3
Fucose	0.201	0.207	0.006	2.821	103
Galactose	0.1998	0.2035	0.004	2.2	101.8
GlcNH	0.2	0.1904	0.006	2.978	95.2
GalNH	0.2	0.1877	0.004	2.01	93.9
Xyl	0.1998	0.2039	0.004	2.109	102.5
NeuAc	29.34	29.56	0.256	0.865	100.7
NeuGc	31.17	30.79	0.167	0.541	98.8

Table 1: Comparison between the concentration calculated by weight and by qNMR of the Monosaccharides Stock solution.

*The concentration calculated by qNMR given is an average of three different NMR samples prepared independently from the stock solution.

Different Quantitative monosaccharide standards have been developed:

•**Monosaccharide mix reference standard** is a quantitative standard comprised of NIST-F and USP traceable **glucosamin**e (GlcN), **galactosamine** (GalN), **galactose** (Gal), **mannose** (Man), **glucose/dextrose** (Glc) and **fucose** (Fuc) monosaccharides. This monosaccharide mix is used as external standard in our **LudgerTag 2-AA Monosaccharide Release and labelling Kit** for a fully quantitation of glycoprotein therapeutics and pre-released glycans**.** Accuracy: The monosaccharide amounts are detailed in Table 2. This analysis was performed on 24 vials.

•**Xylose** (Xyl) quantitative standard of NIST-F and USP traceable Xyl that can be used as an internal standard in in our **LudgerTag 2-AA Monosaccharide Release and Labeling Kit**.

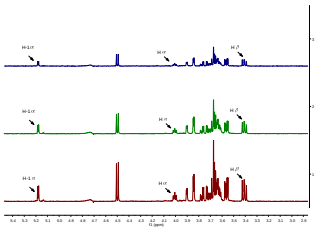


Figure 1. ^{1}H-NMR (500MHz) of **Gal-Bulk** in D_2O. 3 replicates at 3 different concentrations.

Dilution
Mixing
Dispensing
Labelling

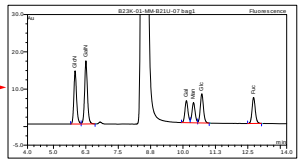


Figure 2. LudgerSep-R2 HPLC profile of 2-aminobenzoic acid (2-AA) labelled mono-mix. (Cat. #: CM-MONO-MIX-10, Batch B21U-07). The peak between 8-9 min is free dye.

Monosaccharide	Catalogue number	Batch number	nmols monosaccharide
GlcN	CM-MONO-MIX-10	B21U-07	9.81 ± 0.13
GalN	CM-MONO-MIX-10	B21U-07	9.96 ± 0.11
Gal	CM-MONO-MIX-10	B21U-07	10.03 ± 0.11
Man	CM-MONO-MIX-10	B21U-07	10.02 ± 0.12
Glc	CM-MONO-MIX-10	B21U-07	10.02 ± 0.11
Fuc	CM-MONO-MIX-10	B21U-07	10.10 ± 0.12

Table 2: Quantitative analysis of the monomix composition. Values are in nmols with a ±95% confidence interval

•**N-Acetylneuraminic acid** (NeuAc) and **N-glycolyneuraminic acid** (NeuGc): quantitative standards of NIST-F and USP traceable NeuAc and NeuGc. They are used as external standard in our **LudgerTag™ DMB sialic acid labelling kit**. Labeling of sialic acids with 1,2-diamino-4,5-methylenedioxybenzene.2HCl (DMB) for fluorescence detection of sialic acid derivatives. Accuracy: The monosaccharide amounts are detailed in Table 3. This analysis was performed on 12 vials.

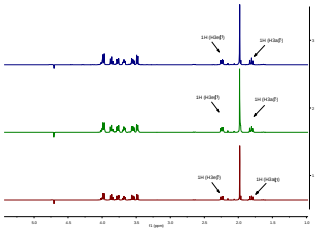


Figure 3. ^{1}H-NMR (500MHz) of **NeuAc-Bulk** in D_2O, 3 replicates.

Dilution
Dispensing
Labelling

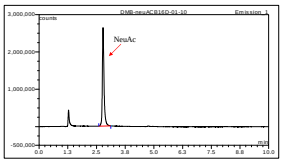


Figure 4. LudgerSep-uR2 HPLC profile of 1,2-diamino-4,5-methylenedioxybenzene.2HCl (DMB) labelled NeuAc standard (Cat. #: CM-NEU-AC-01, Batch B16D-01)

Monosaccharide	Catalogue number	Batch number	nmols monosaccharide
NeuAc	CM-NEU-AC-01	B16D-01	1.11 ± 0.02
NeuGc	CM-NEU-AC-01	B16E-08	1.04 ± 0.01

Table 3: Quantitative analysis of the NeuAc and NeuGc standards. Values are in nmols with a ±95% confidence interval

BioQuant Glycan Standards: Monosaccharide analysis vs qNMR

Isolated Glycans from complex natural Source or Protein → **How much???** → **Monosaccharide analysis** / **qNMR** → **Agree** → **Bioquant Glycan Standards**

Quantification of Chitotriose

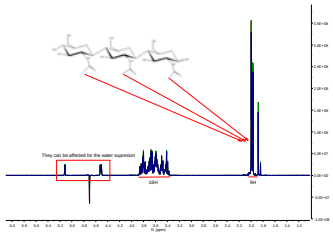


Figure 5. ^{1}H-NMR (500MHz) of **chitotriose** in D_2O, 3 replicates. Samples at same concentration

	A*: Concentration by monosaccharide analysis (mM)	Std dev	CV	B: Concentration using by qNMR (mM)	A/B Difference
Chitotriose-1	4.19	0.057	1.35	4.3	97.57
Chitotriose-2	4.71	0.147	3.12	4.85	97.16
Chitotriose-3	5.07	0.058	1.14	5.18	97.92

Table 4. Comparison between the concentrations calculated by monosaccharide analysis and by qNMR of three samples of chitotriose at different concentrations. *The concentration calculated by monosaccharide analysis given is an average of four different replicates.

	A: Concentration by monosaccharide analysis (mM) in 10 µl of sample	B: Concentration using by qNMR (mM) in 10 µl of sample	Std dev	A/B Difference
GPEP-A2	3.82	3.85	0.003	101.54

Table 5. Comparison of concentration of GPEP-A2 calculate using Monosaccharide analysis and qNMR.

Quantification of GPEP-A2

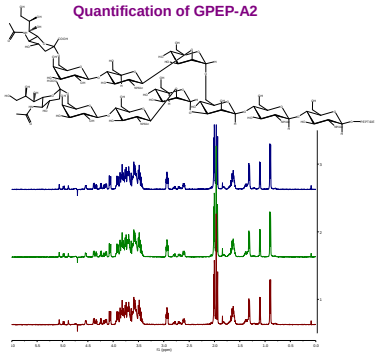


Figure 6. ^{1}H-NMR (500 MHz) of **GPEP-A2** in D_2O. Samples 1-3

Conclusion

Orthogonal quantitative determination techniques have been successfully utilized to confirm the absolute quantities of monosaccharide standards in both the **LudgerTag 2-AA Monosaccharide Release and Labelling Kit** and **LudgerTag™ DMB sialic acid labelling kit**. This level of precision and accuracy has also been applied to complex glycans and glycopeptides. Through the use of better standards, glycoanalysts in industry and academia can gain ever more reliability in the characterisation of their glycoproteins products.

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