Ludger Guide to Sialylation: II

Highly Sialylated Glycoproteins

Ludger has over 15 years' experience providing products and services for the biopharmaceutical industry and in that time we have noticed that in addition to monoclonal antibodies (which have little or no sialylation on their Fc portion), many more sialylated glycoproteins are regularly being developed. Examples include Fc fusion proteins, hormones (such as EPO), vaccines and clotting factors. Since sialic acids can influence drug safety and efficacy, regulatory bodies are demanding more rigorous characterisation of glycans. With the widespread development of biosimilars by the industry, this has been highlighted by the new EMA Guideline on Biosimilars Quality which requires that extensive state-of-the-art characterisation studies are performed in parallel on both reference medicinal product and the biosimilar (see Reference below).

Glycan sialylation is important for the safety and efficacy of a drug

In humans the major sialic acid on glycans is Neu5Ac, however Neu5Gc (which has a glycolyl group instead of an acetyl) can be added to glycoproteins that are expressed in different cell lines. This non-human Neu5Gc may elicit an immunological response which can lead to inflammation and/or neutralisation (and therefore loss of efficacy) of the biopharmaceutical. Sialylation of glycans can also have a major effect on the pharmacokinetic properties of a drug: for example, glycans with terminal galactose (without sialic acids) are removed from circulation by the asialoglycoprotein receptor in the liver. Glycoproteins such as EPO can have sialic acids with extra acetylation (e.g. Neu5,9Ac_2) which may affect drug efficacy. Sialylation can also be important for receptor binding and signal transduction; for instance the presence of sialic acid on IgG Fc glycans reduces ADCC (Antibody Dependent Cellular Cytotoxicity) activity and is anti-inflammatory.

How can Ludger help?

There are a number of approaches that can be used to aid the characterisation of sialylated glycans using validated Ludger technology.

1. Identification and quantification of sialic acids:

a) Ludger DMB Sialic Acid Labelling Kit (Cat# LT-KDMB-A1) to release, identify and obtain relative quantitation of Neu5Ac, Neu5Gc and Neu5,9Ac_2 by (U)HPLC analysis. We use the fluorescent tag DMB to specifically label sialic acids, then use (U)HPLC with fluorescent detection to detect and quantitate them (see Figure 1) The use of a fluorescent tag makes the assay more sensitive than PAD. As the DMB tag only becomes fluorescent when labelling the sialic acids (across 2 adjacent keto groups), this specificity also overcomes issues due to other components in the sample buffer (which can be a concern with PAD detection).

b) The absolute amounts of Neu5Ac and Neu5Gc on a glycoprotein can also be determined by comparison to Ludger quantitative standards (Cat # CM-NEUAC-01, CM-NEUGC-01).

c) Ludger's quantitative sialylated glycopeptide standard (Cat # BQ-GPEP-A2G2S2-10U) is recommended to check the efficiency of glycan release, labeling and recovery and will give you confidence in the accuracy of your sialic acid measurements.

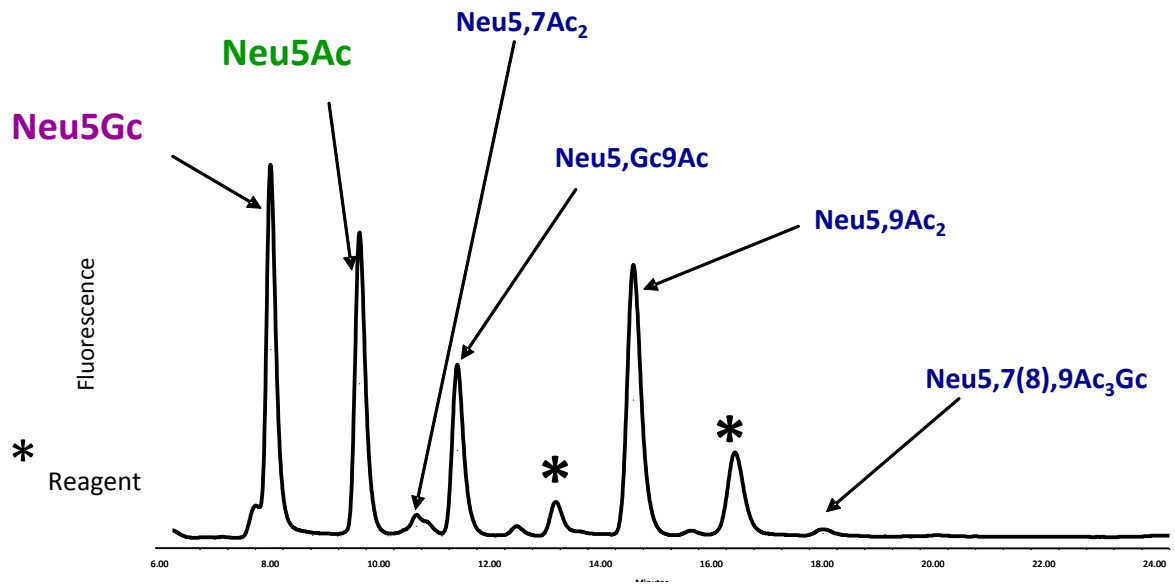

Figure 1: RP-HPLC Analysis of DMB Fluorescent Labelled Sialic Acids (released by mild acid hydrolysis) from the Sialic acid Reference Panel. This Reference Panel is part of the LT-KDMB-A1 kit

2. Analysis and characterisation of sialylated glycans:

N-glycans are removed from the biopharmaceutical by PNGase F (Cat # E-PNG-01). The glycans are fluorescently labelled with 2AA or 2AB using using LudgerTag kits incorporating 2-picoline borane reductant (Cat # LT-KAB-VP24 or LT-KAA-VP24) and cleaned up using LC-T1-A6 cartridges.

a) Analysis by HILIC- (U)HPLC provides GU values which can be matched to database and standard GU values to obtain preliminary structure assignments.

b) Further analysis following exoglycosidase sequencing can be used to characterise the structures present (Figure 2). For example peaks that disappear (or reduce) following digestion with sialidase (Cat# E-S001) will have contained sialylated structures.

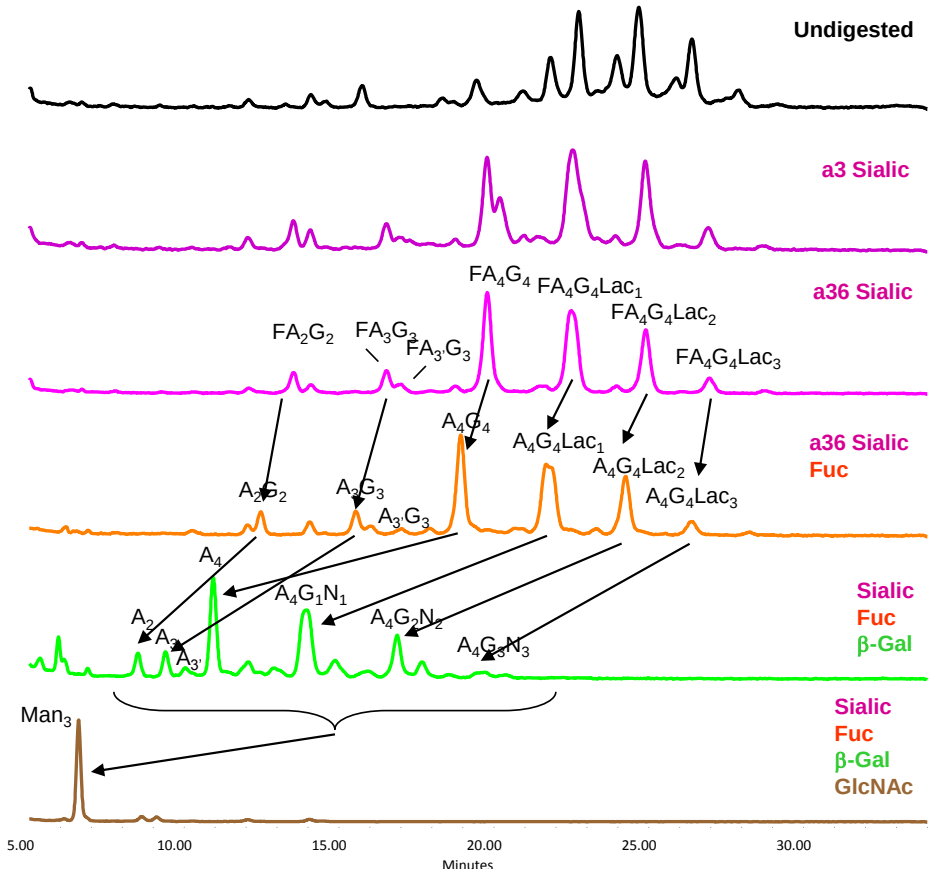

Figure 2: Exoglycosidase sequencing of EPO; 2AB labelled glycans run on a LudgerSep-N2 Column
Enzyme specificities: a3Sialic = α1-3 sialic acid; a36Sialic = α1-3 & 6 sialic acids; Fuc = α1-6 fucose; bGal = β1-4 galactose; GlcNAc = β1-2,4,6 N-acetyl-glucosamine.

Structure names: F indicates a core fucose; Mx, number (x) of mannose on core GlcNAcs; Ax, number of antenna (GlcNAc) on trimannosyl core; A2, biantennary with both GlcNAcs as β1-2 linked; A3, triantennary with a GlcNAc linked β1-2 to both mannose and the third GlcNAc linked β1-4 to the α1-3 linked mannose; A3', triantennary with a GlcNAc linked β1-2 to both mannose and the third GlcNAc linked β1-6 to the α1-6 linked mannose; A4, GlcNAcs linked as A3 with additional GlcNAc β1-6 linked to α1-6 mannose; Gx, number (x) of linked galactose on antenna; Lac x, number (x) of lactosamine repeats; N x, number(x) of terminal GclNAcs.

c) Charge separation of labelled glycans on a LudgerSep-C3 weak anion exchange (WAX) column can provide data on the relative proportions of the mono-, di-, tri- and tetra-sialylated glycans. (Figure 3).

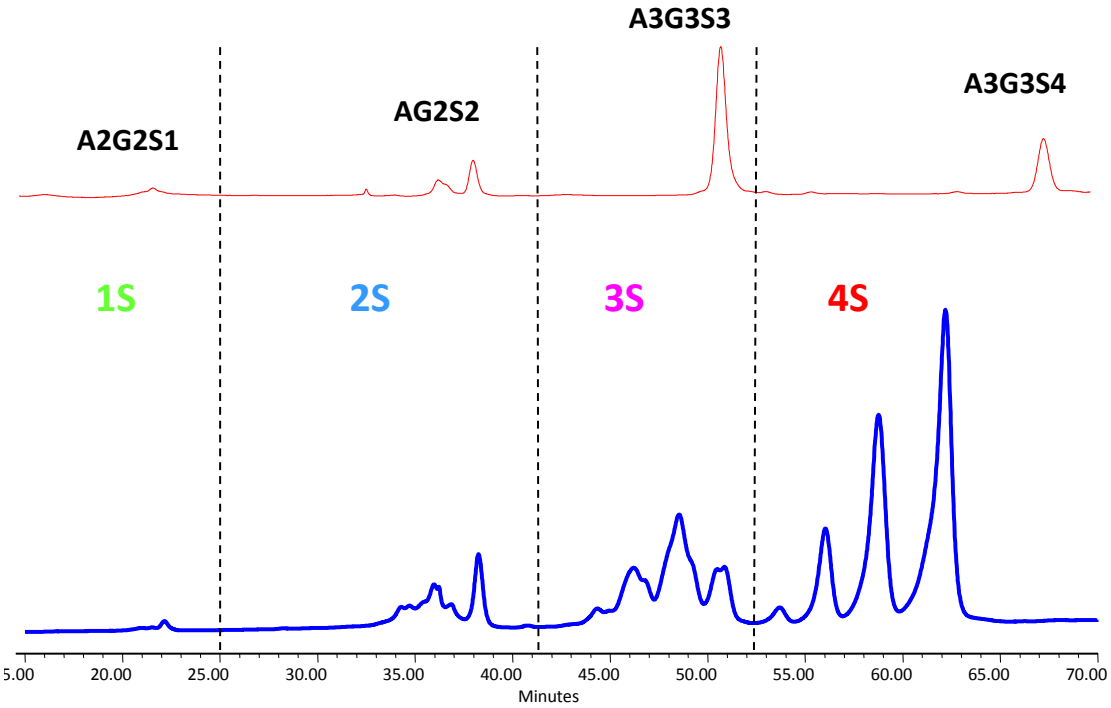

Figure 3: Weak anion exchange (WAX)-HPLC Charge separation of 2AB labelled Glycans on a LudgerSep-C3 column. Top trace (red): fetuin N-glycan. Bottom trace(blue): EPO N-glycans

For complex mixtures, these differently charged fractions can be separated and analysed further by HILIC-(U)HPLC with exoglycosidase sequencing to fully characterise the structures (Figure 4):

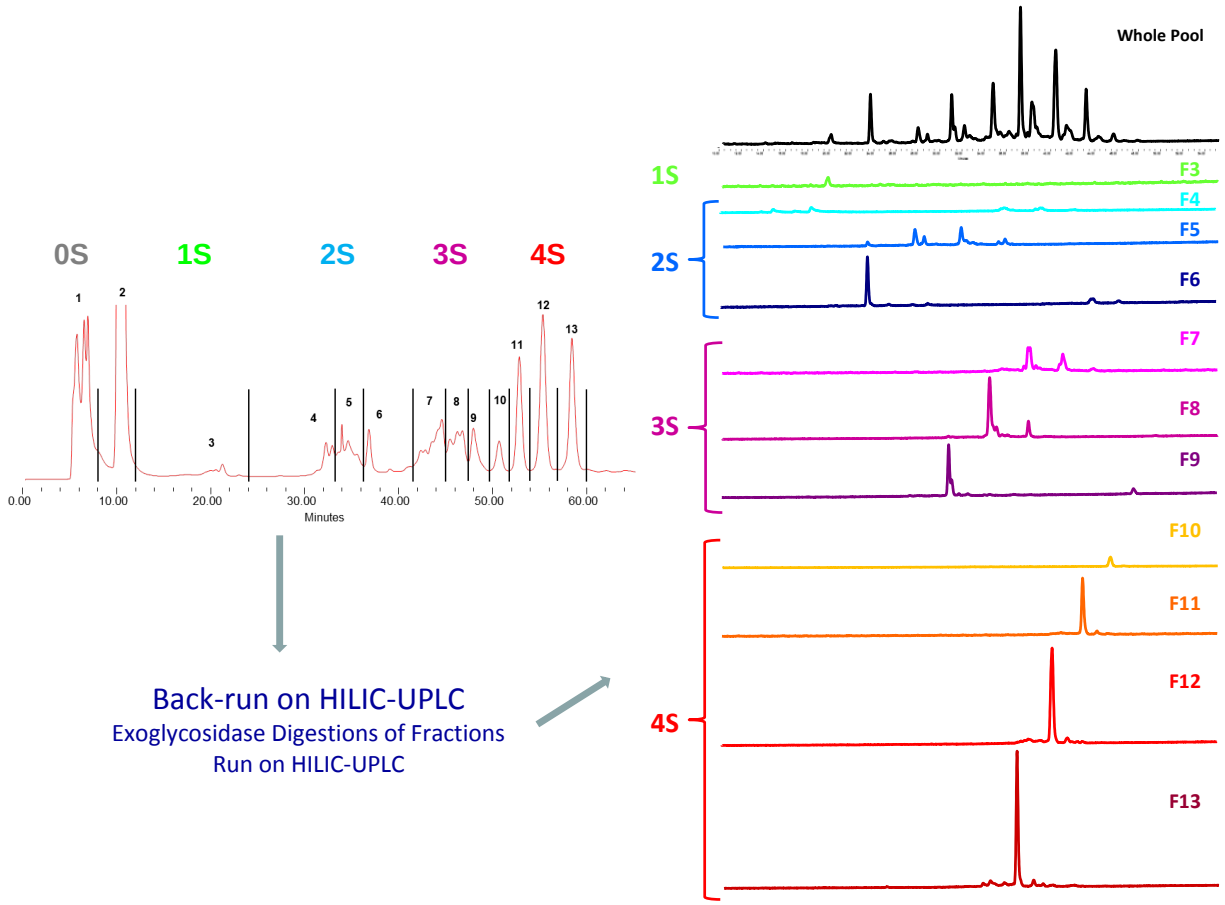

Figure 4: Charge fractionation of EPO N-glycans by WAX-HPLC followed by HILIC-UPLC.

d) Mass composition data can be obtained by MALDI analysis following permethylation to stabilise the sialic acids (Figure 5).

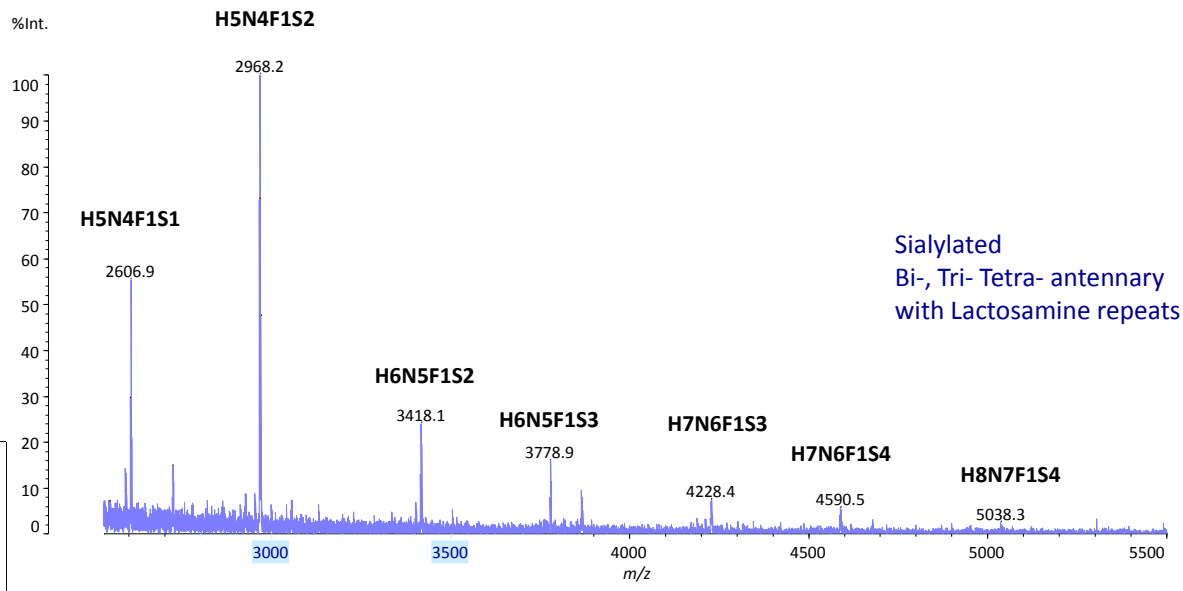

Figure 5: MALDI analysis of permethylated EPO N-glycans

If you require any further information on how we can help you with selection of products (for your in-house analyses), Ludger services (our in-house analyses) or method transfer, please contact us at info@ludger.com

Reference: "EMA Revises Biosimilars Guideline on Quality Issues" in Hogan Lovells (ed) Focus on Regulation, posted on June 9, 2014 by Elisabethann Wright and Ciara Farrell.