

NPL REPORT AS 6

Characterisation and Quantitation of Post- Translationally Modified Proteins

**Smita Thobhani, Chun-Ting
Yuen, Chris Jones, Marc
Bailey**

NOT RESTRICTED

JUNE 2007

Characterisation and Quantitation of Post-Translationally Modified Proteins

Smita Thobhani, Marc Bailey
Quality of Life Division, NPL

Chun-Ting Yuen, Chris Jones
National Institute for Biological Standards and Control

ABSTRACT

This report describes work carried out under the Measurements for Biotechnology (MfB) Programme 2004-2007, for addressing comparability issues in glycan analysis when monitoring protein glycosylation.

An inter-laboratory study was undertaken within the UK to assess the comparability of glycan analysis across multiple platforms. The study consisted of a questionnaire and a laboratory based study, in which participants analysed a set of four *N*-glycans using their routine glycan mapping methodology. Results from the study are to be used to help harmonise the field of glycan analysis and to support writing of pharmacopoeial monographs.

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ISSN 1754-2928

National Physical Laboratory
Hampton Road, Teddington, Middlesex, TW11 0LW

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1.0 INTRODUCTION

This report has been produced as part of the “Measurement for Biotechnology” programme (2004-2007): *More comparable measurement of higher order structure and interactions of proteins*; and is supported by the Department of Trade and Industry, under the National Measurement System. The programme aims to improve the comparability measurements of the UK industry in the area of Biotechnology. Further information about this project, and others under the programme, can be obtained at: <http://www.mfbprog.org.uk> or <http://www.npl.co.uk/biotech>.

1.1 Scope

This project addresses comparability issues in glycan analysis when monitoring protein glycosylation. The project was led and coordinated by the National Physical Laboratory (NPL) with the National Institute for Biological Standards and Control (NIBSC). The involvement of NIBSC was fundamental for the scientific direction of the project and to ensure that the outputs from it could be correlated to other investigations in glycan analysis. The high degree of industrial participation in the inter-laboratory study demonstrates the relevance of this work in the field of protein glycosylation. The areas that this project will disseminate to are:

- The national and international regulatory community through the regulatory role of NIBSC and through the work of USP on glycan analysis.
- The UK biopharmaceutical industry through the participation of various industrialists in the comparison study
- The UK analytical services industry through participation in the comparison study of various laboratories that provide analytical services to the UK biopharmaceutical sector.

1.2 Regulatory aspects of glycoprotein glycan analysis

As the number of glycoprotein therapeutics undergoing clinical trials and reaching the market increases, so does the awareness of the importance of detailed analysis of the oligosaccharides attached to the protein; particularly as changes in glycosylation during production can significantly affect the biological activity of a therapeutic molecule.

Competent authorities who license products work to international guidelines developed by the International Conference on Harmonisation (ICH) (see <http://www.ich.org>), who indicate the level of characterisation required and the level of validation of that analytical methodology. Regulatory agencies in Europe (EMA) and the USA (FDA) also provide more detailed guidance to manufacturers about their expectations. The marketing approval will detail the full range of in-process and final product tests, which must be carried out to allow batches of product to be sold. The purpose of these in-process and lot-release tests is to demonstrate consistency of the individual batches with the material used in clinical trials, in terms of quantity, integrity and potency, product profile, process-related impurities and sterility.

When more than one manufacturer is producing a product, pharmacopoeial monographs are written by the European Pharmacopoeia (EP) or the United States Pharmacopoeia (USP), which detail a minimal set of tests that must be carried out, and general chapters providing additional guidance or detailing robust methodology may be included.

Current guidelines from the ICH covers glycan characterisation during/from production (section Q6B; updated 1999), and after manufacturing changes (Q5E; updated 2004). The structural characterisation of carbohydrates under Q6B is quoted as: *“For glycoproteins, the carbohydrate content (neutral sugars, amino sugars, and sialic acids) is determined. In addition, the structure of the carbohydrate chains, the oligosaccharide pattern (antennary profile) and the glycosylation site(s) of the polypeptide chain is analyzed, to the extent possible.”*

Regulatory agencies are currently struggling to define appropriate measures of similarity, which can be used to license “biosimilar” products (called “follow-on products” in the USA); particularly as glycosylation is rather variable (depending as it does on the cell line and culture conditions) and its role in modulating the biological activity *in vivo* may be undefined. Thus, an understanding of the variability of the analytical methods used is required to underpin these decisions.

Current pharmacopoeial monographs are limited to product-specific tests to analyse the composition of intact glycoproteins, and charge-based separation methods to determine the degree of sialylation. Generally a case-by-case approach has been taken for different glycosylated products, for example the EP monograph for recombinant erythropoietin (rhEPO), which includes a capillary zone electrophoresis assay to look at different sialylated glycoforms. General monographs relating to the analysis and profiling of cleaved glycans are not currently available despite this approach being the common one used for characterising *N*-glycans. Therefore there is a need to update the monograph to reflect current trends in glycan analysis with better guidance on the techniques to employ, along with the guidance on the level of characterisation required at various stages of production.

Both the EP and USP are developing general chapters for glycoprotein and glycan analysis, and there intends to be a high level of harmonisation between these monographs. Both organisations will produce an introductory monograph detailing the needs for glycan analysis and an overview of methodology (to be called <1084> by the USP), and additional methodological monographs. For example, USP <1095> will detail oligosaccharide analysis methods, with additional ones on the deglycosylation of glycoproteins (USP<1085>) and on monosaccharide analysis (USP<1094>).

1.3 Project aims

Despite a range of methodologies which exist to analyse protein glycosylation, the challenges and complexity of characterising glycans make this field a multi-method area, and as a consequence comparability issues across various platforms arise. In addition, the advances in analytical techniques to elucidate the structures of glycans also place a greater emphasis on providing detailed glycan analysis. Thus there is a need to assess the comparability of the different glycan analysis methodologies with the aim of harmonising the field to support regulatory approval.

Correlation of the key structural characteristics of glycans with data from different techniques utilised in analysing them will provide, an understanding of the variability issues that can arise when comparing data from multiple platforms. This project aimed to address some of these issues with an inter-laboratory study. The study was composed of two components, a questionnaire and a laboratory based study. The questionnaire was designed to find out about the methods employed by participants in the laboratory study. The laboratory study involved participants around the UK analysing samples of *N*-glycan chains using their routine methodologies; the amount of information obtained, its variation and comparability across the methods utilised was then assessed. Data obtained from the participants was anonymised with only NPL and NIBSC having access to the original data set.

It was envisaged that the study would provide an insight into whether the different methodologies carried out in the different laboratories generated comparable data, and enable the resolution of these approaches with respect to their ability in differentiating different *N*-glycan structures to be assessed. The range of experimental data for quantification of individual structures was also to be considered. The study was designed to enable the commonly used techniques to be highlighted, and thus help prioritise and focus future development of standards in this area.

Information from the study will be transferred to a larger, international, glycan analysis inter-laboratory study that NIBSC are coordinating with USP. The findings from both this study and USP's investigation will then be combined; to help inform the writing of monographs in the USP and EP, on general methodology based chapters for the analysis of cleaved glycans. In addition to using these studies there will be contribution from a Measurement for Biotechnology validation project, undertaken by LGC with NIBSC, on glycoform microheterogeneity [1].

2.0 BACKGROUND

2.1 Post-translational modifications

The majority of natural and recombinant proteins undergo some form of either enzymatic or chemical post-translational modification (PTM). These modifications can profoundly affect the immunogenicity, protein folding, stability, cell signalling, pharmacokinetics and the efficacy of a protein. In addition to understanding the biological influence that these PTMs bestow, there is the added problem of identifying and characterising these changes for the regulatory approval of a therapeutic protein. Correctly detecting PTMs in products may lead to modifications in the manufacturing process, to minimise undesirable modifications and improve yields.

Proteins can be subjected to a broad range of modifications such as those portrayed in Table 1. Some can also be synthetic like PEGylation and fluorescent/radiolabelling. Exposure to glucose or urea during cell culture and processing can respectively, result in glycation and carbamylation of proteins, respectively. However, despite all the different protein modifications that can occur the most commonly encountered one is glycosylation.

Table 1: Post-translational modifications commonly found on proteins

Type of PTM	Description
Acetylation	Addition of an acetyl group
Acylation	Hydrolysis of methionine residue at the N-terminus followed by the addition of an acyl group
Amidation	Breakdown of glycine residue at the C-terminus to leave an amino group
Carboxylation	Conversion of glutamate to γ -carboxyglutamate <i>via</i> the co-factor vitamin K
Deamidation	Conversion of glutamine/asparagine to glutamic/aspartic acid
Disulphide bonds	Covalent linkage of two cysteine residues
Glycosylation	Addition of carbohydrates to e.g. asparagine, serine, threonine
Hydroxylation	Proline and lysine hydroxylation <i>via</i> the co-factor vitamin C
Methylation	Addition of a methyl group to e.g. lysine, arginine
Phosphorylation	Addition of a phosphate group to e.g. serine, tyrosine, threonine, histidine
Prenylation	Addition of isoprenoid groups to cysteine residues at the C-terminus of proteins in a thioether linkage
Proteolysis	Cleavage of a protein at a peptide bond
Sulphation	Addition of a sulphate group to e.g. tyrosine

2.2 Protein glycosylation

Protein glycosylation is the covalent attachment of carbohydrate (oligosaccharide) moieties to proteins to form glycoproteins. In mammalian systems the attachment of oligosaccharides to proteins arises in three ways: either through an amide group on asparagine (Asn) (*N*-linked), a hydroxyl group on serine (Ser) or threonine (Thr) (*O*-linked) or a glycosylphosphatidylinositol (GPI) lipid anchor on the carboxy-terminus of a protein. The oligosaccharides are composed of various combinations of some of the following commonly occurring monosaccharides: mannose (Man), glucose (Glc), *N*-acetylglucosamine (GlcNAc), galactose (Gal), *N*-acetylgalactosamine (GalNAc), fucose (Fuc), inositol (Ins) and *N*-acetylneuraminic acid (Neu5Ac - also known as sialic acid) (Figure 1).

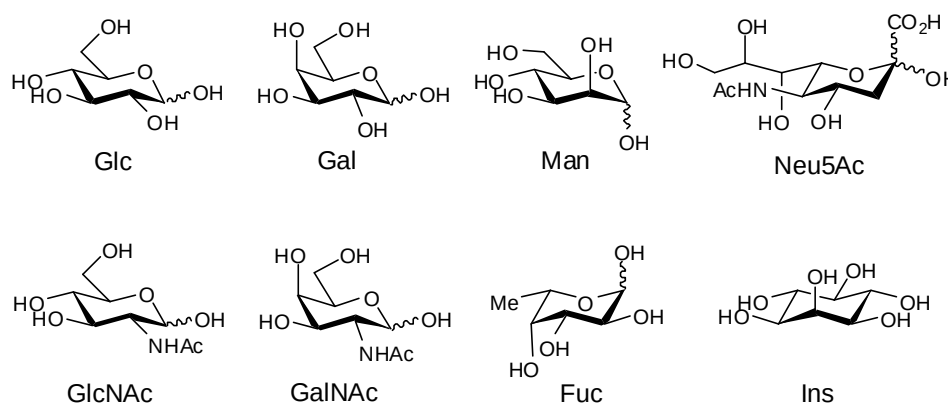


Figure 1: Structures of the commonly occurring monosaccharides

The extent of glycosylation varies from protein to protein and can depend on the size of the protein and the source and cell-type of the protein.

2.2.1 *N*- and *O*-Glycans

N-Linked glycans are located on Asn residues when the amino acid appears in the sequence Asn-X-Ser/Thr, where X can be any amino acid besides proline and aspartic acid. The structures of *N*-glycans can be subdivided into three types: high mannose, hybrid and complex (Figure 2), each containing a common core pentasaccharide at the “reducing end”, consisting of three mannoses and two *N*-acetylglucosamine residues ($\text{Man}_3\text{GlcNAc}_2$) [2]. In complex type chains the degree of branching (i.e. number of antennae) typically varies, for instance bi-, tri- (2,6-branched illustrated) and tetra-antennary structures can be produced with differing amounts of “outer arm” sugars such as fucose, bisecting *N*-acetylglucosamine and sialic acid. Glycosylation patterns observed in *N*-glycans structures are that the antennae usually consists of repeating units of mannose (as in the case for high-mannose glycans) or, alternatively, one or more *N*-acetylglucosamine units (GlcNAc) attached to mannose residues. Here the oligosaccharide chains are frequently terminated with sialic acid residues (*N*-acetylneuraminic acid), the prevalent acidic sugar. In some instance poly-*N*-acetylglucosamine sequences can be found.

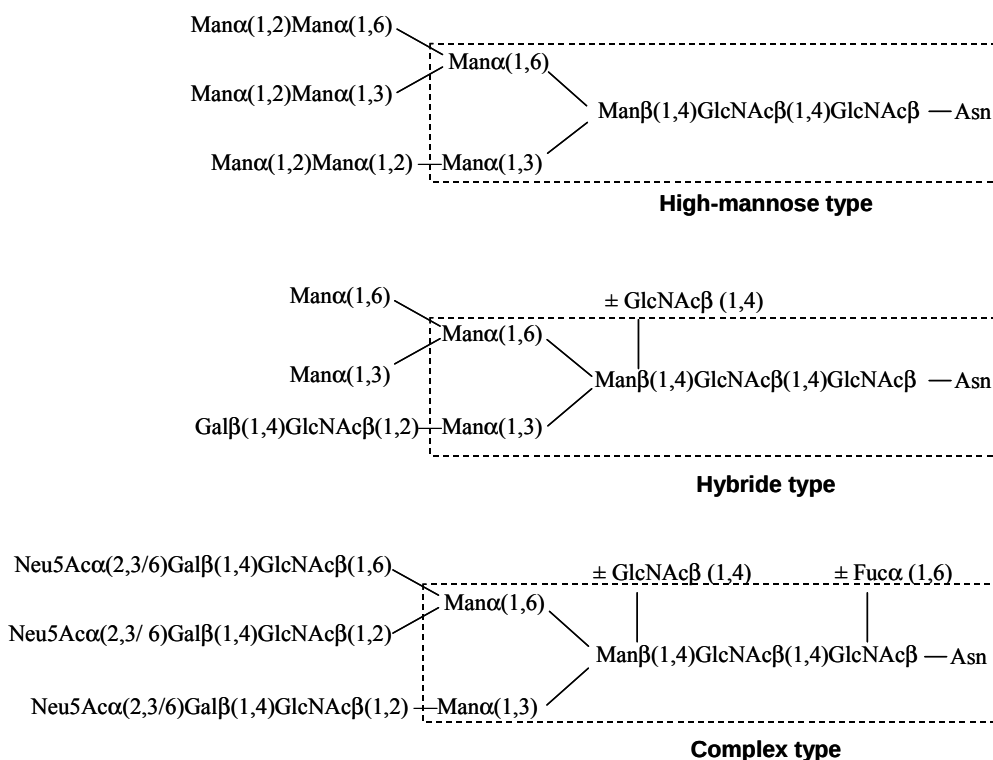


Figure 2: The three common types of N-glycans found on mammalian proteins with the core pentasaccharide inside the box

The presence of a core pentasaccharide in the three N-glycan types can be ascribed to the fact that during their biosynthesis all three types originate from the same precursor containing this core pentasaccharide and some additional sugar residues. During enzymatic processing, which takes place in the endoplasmic reticulum (ER) and Golgi complex, this precursor is altered to yield high mannose, hybrid and complex type N-glycans (Figure 3) [3].

Briefly, synthesis begins on the cytoplasmic surface of the ER membrane with the attachment of N-acetylglucosamine to dolicholphosphate (Dol-PP) with subsequent addition of an N-acetylglucosamine and five mannoses to the molecule step-by-step using glycosyltransferases to yield Man₅GlcNAc₂ (Figure 3) [4]. This structure is then flipped to the luminal side of the ER membrane where four further mannoses and three glucoses are enzymatically added. The Dol-PP is then released and the oligosaccharide chain transferred to the asparagine residue of a polypeptide chain that is in the process of folding. Upon folding of the glycoprotein some of the sugar residues (three glucoses and one mannose) are now trimmed away by glycosidases [5] and the glycoprotein is then transferred to the Golgi complex [6]. Here additional modifications take place including further trimming of mannoses, the addition of N-acetylglucosamines and galactose residues, followed by the addition of fucose and sialic acid molecules *via* numerous different enzymes. The final glycoforms present, and therefore functional activity, will differ depending on the tissue and the cell in which the glycoprotein was expressed.

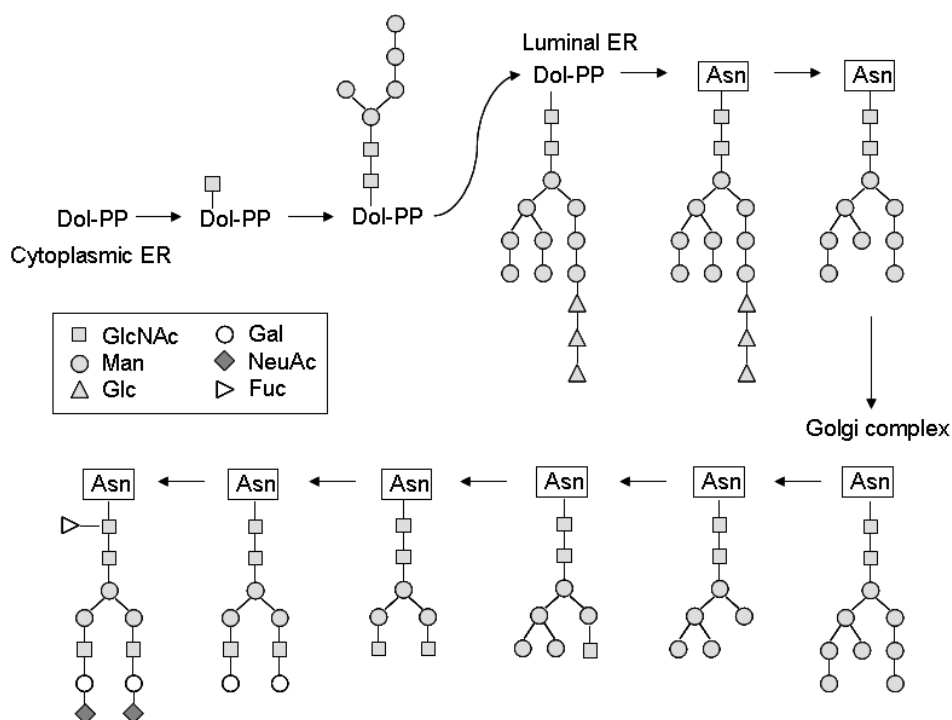


Figure 3: Biosynthesis of N-glycans [7]

O-Glycans are typically linked to the protein *via* an *N*-acetylgalactosamine sugar but unlike *N*-glycans the sequence of monosaccharides and linkage determination are more complicated as there is no single common core structure, and at least eight types have been identified so far (Figure 4). Furthermore, there are two common forms of O-glycosylation: one has relatively few scattered O-glycans that can be either short chains with few residues, or as elongated structures similar to *N*-glycans but less branched. The other form, the mucin-type, occurs when a large number of serine and threonine residues are present resulting in heavily clustered O-glycosylation to give gelatine like molecules, which usually form part of the cell surface extra-cellular matrix or secreted glycoproteins [3].

There are other unusual O-glycosylations such as O-GlcNAc found on many nucleocytoplasmic proteins [8], O-fucose and O-glucose linked glycosylation on epidermal growth factor-like serum proteins [9] and O-mannose linked glycosylation in some muscular and neural glycoproteins [10] and in yeast [11]. Further details of the nature, biosynthesis and distribution of *N*- and *O*-linked glycans can be found in the review by Spiro [12] and in Essentials of Glycobiology [3].

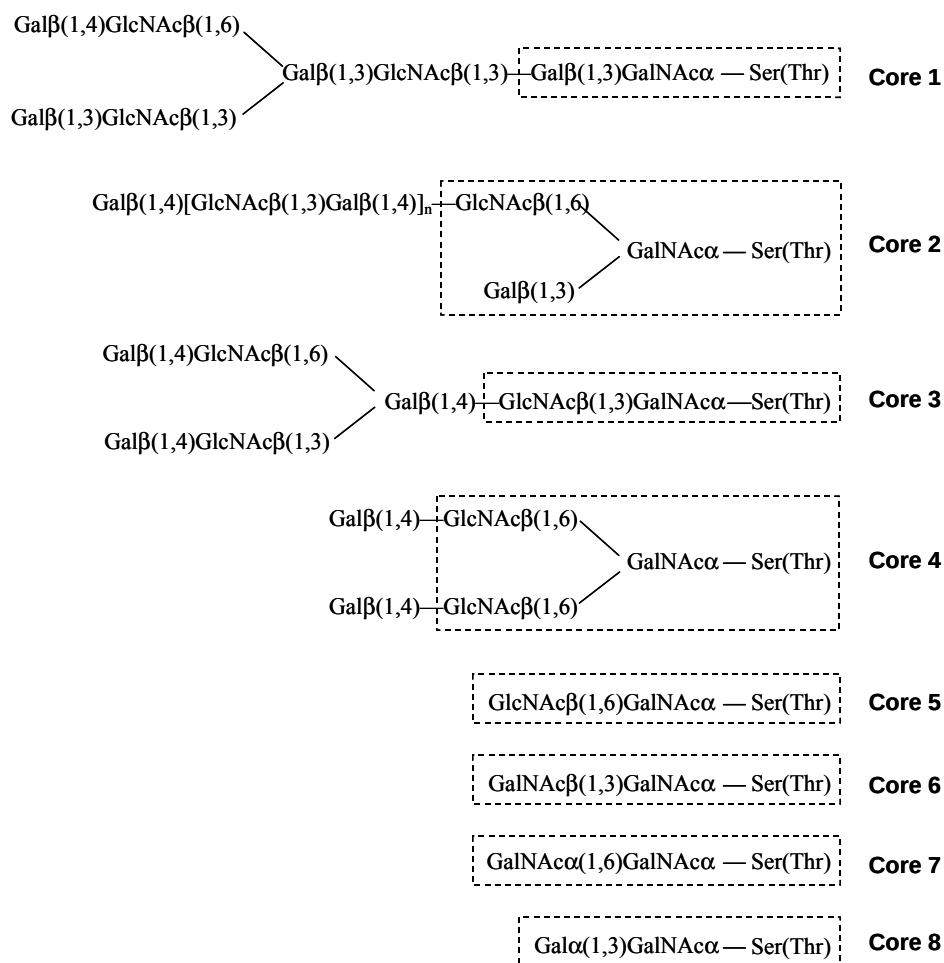


Figure 4: Typical O-linked glycan core structures

2.2.2 Heterogeneity

Not only can the degree (fully, partially or un-occupied), type (*N*- or *O*-linked) and the site of glycosylation vary from protein to protein, but the actual oligosaccharide structures also differ from glycosylation site to glycosylation site and even on the same site. Variations in the glycan branching and linkages at any given glycosylation site in a protein can also be found. The structural variations found in glycans at any given glycosylation site on a glycoprotein is termed “microheterogeneity”. This heterogeneity results in a single species of glycoprotein having numerous glycoforms, each one a different molecule; in other words any glycoprotein can consist of a mixture of a single peptide backbone with one or more of about a hundred different glycan chains attached. This heterogeneity arises because, unlike the protein or DNA sequence of these molecules, glycosylation is a non-templated process. The specific glycosylation pattern at a given site depends on many factors, including the cell-specific and/or growth-dependent availability of glycosyltransferases and exo-glycosidases found in the Golgi bodies and ER, which influence the terminal glycan sequences: the region where the greatest variation is encountered. Heterogeneity leads to different physical and biochemical properties and, therefore, also to functional diversity.

2.3 Biological significance of protein glycosylation

It is estimated that over 50 % of human proteins are glycosylated and thus, it is not surprising that carbohydrates participate in a plethora of biological events, for which there are many excellent reviews [12-17]. In brief, the biological role of glycosylation can be grouped into two general categories: the specific recognition/interaction of glycans with receptors (e.g. lectins, antibodies, enzymes), and the modulatory and structural properties of the molecules to which the glycans are attached such as providing specificity and stability.

As expected there is an array of carbohydrate-receptor interactions that occur in nature, many of these involve cell adhesion (bacterial, viral, toxins) [18], cell (leukocyte) trafficking [19, 20], cell-cell interactions [21], endocytosis, the immune system (immunoglobulins and complement components) [22-25] and cell signalling events. The glycans involved in these recognition/binding events are often encountered on cell surfaces attached to proteins. A classical example is the adhesion of selectins on endothelial cells to carbohydrates found on the cell surfaces of white blood cells in inflammation [26]. The influenza virus infects its host by initially targeting the sialic acids on glycoproteins found on cell surfaces with the protein haemagglutinin followed by its release with neuraminidase [27]. Sperm-egg binding is triggered by the interactions of the *O*-glycans on the egg zona pellucida glycoprotein [21]. Tumour cells often display unusual sugars on their surface that can be exploited as targets for antibody production to aid in the destruction of the cells.

Structurally, glycans on glycoproteins are capable of providing a basic “shield” by protecting the protein from proteases and recognition from antibodies. They also enable the polypeptide chains in the ER to fold in the desired manner and help maintain the correct conformation and solubility of the protein [6, 28, 29]. A protein that is incorrectly glycosylated or where glycosylation has been inhibited will misfold and even aggregate resulting in the failure of the protein to reach its functional state and leave the ER. Carbohydrates can also act as “tags” for the proteins to which they are attached by helping them relocate from the Golgi to specific compartments in cells or to the cell surface. Aside from structural roles, glycans can exhibit modulatory functions in the interactions of one protein to another [30], for instance successful binding of growth factor receptors is dependent upon glycosylation. In the case of the hormone β -human chorionic gonadotrophin, binding of the deglycosylated material to its respective receptor is attainable but subsequent activation events arising from binding are absent.

Defects in the genes involved in protein glycosylation pathway or in the degradation of glycoproteins ultimately alters the function of nearly all organ systems, causing disorders with medical consequences [31]. Many autoimmune diseases [24, 32] and cancer [33] are associated with defined changes in glycosylation. Carbohydrate-deficient glycoprotein syndrome (CDG) is a congenital disorder caused by incomplete or failed production in the early steps of *N*-glycan biosynthesis. Infants with the disease have multi-system failures including neurological abnormalities, skeletal irregularities, lipodystrophy and blood coagulation defects [34-36]. Malfunctioning along the degradation pathway, which is mediated by *exo*- or *endo*-glycosidases, results in the premature termination of the process with an accumulation of the non-degraded material inside the lysosome causing lysosomal storage disorders [37].

2.4 Therapeutic glycoproteins

Understandably, multi-functional glycoproteins are of great interest to the pharmaceutical industry either as drug targets, mediators, diagnostic markers for diseases, or as therapeutic products themselves - the characterisation of which is the subject of this report.

Therapeutic glycoproteins are mostly recombinant DNA products produced in cell culture systems or in transgenic animals, and represent a global biopharmaceutical market worth over a third of the billion dollar marketplace [38]. Glycoproteins include monoclonal antibodies (mAbs), rhEPO, granulocyte macrophage-colony-stimulating factor (GM-CSF), interferons (IFNs) and tissue plasminogen activator [39, 40]. The top-selling protein therapeutic is indeed a glycoprotein: Amgen's Aranesp, a re-engineered variant of the company's first-generation recombinant human erythropoietin product, Epogen. Additionally, approximately 80 recombinant glycoproteins are currently under development. The presence (or absence) of carbohydrates is known to have dramatic effects on stability, folding, activity, half-life during circulation and immunogenicity in many therapeutics [41], and so not surprisingly controlling and monitoring glycosylation is of major importance during their development and production. In many cases, optimisation of glycosylation is desired to prolong the circulatory half-life in the blood of the drug. Targeting specific tissues and cell types through the manipulation of glycosylation has also been an element of drug design for therapeutics.

Glycoproteins under development or already approved can generally be broken down into three main categories: i) recombinant mAbs, ii) recombinant blood proteins and iii) recombinant hormones and cytokines. MABs are the most abundant group of products under development. To date, all approved recombinant mAbs have been from the sub-class immunoglobulin G (IgG); a molecule that has two light chains and two heavy chains intertwined covalently and non-covalently to form two antigen binding regions (Fab) and one constant (Fc) region. It is within this Fc region at the amino acid Asn₂₉₇ that *N*-linked complex bi-antennary glycan chains are found (Figure 5). These chains are predominantly asialylated, bearing a mixture of agalactosyl, one or two terminal galactose residues and are, on occasions, bisected with *N*-acetylglucosamine and/or a fucose unit at the reducing end of the chain.

These *N*-glycans enables the Fc portion of the IgG molecule to maintain its structure and thus have the correct conformation so that effector ligands can bind and, for example, activate clearance mechanisms to destroy the antigen and its carrier [42, 43]. This process is known as 'antibody dependent cell cytotoxicity' (ADCC) and is sensitive to the composition of *N*-glycans and in their absence the antibody can bind to antigen but do not elicit an ADCC response. In addition, variable glycosylation modulates the binding of complement and activation of complement-mediated pathways for killing/invading microorganisms. The specific roles of the sugars in maintaining and modulating effector functions in IgG of physiological relevance have been recently reviewed [44], as has the biological roles of the IgG subclasses [45].



Figure 5: Glycosylated Fc region of IgG, crystal structure (Krapp *et al.* [46])

The heterogeneity observed in mAbs can vary widely from clone to clone and is dependent on the mode of production and the culture conditions [47]. Under non-optimal conditions a number of abnormally glycosylated materials are produced, lacking in potency and potentially immunogenic and rendering them unsuitable for human use.

Products that fall within the hormone and cytokines class include EPO, follicle stimulating hormone (FSH), luteinizing hormone (LH), choriogonadotrophin hormone (CG), GM-CSF and interferon-beta (IFN- β); all have multiple *N*-linked and/or *O*-linked chains. An example where appropriate glycosylation is critical for protein activity is the blockbuster drug EPO used in the treatment of anaemia and in cancer patients. EPO is a haematopoietic cytokine growth factor that is naturally produced in the kidney to induce proliferation and differentiation of erythroid progenitor cells thus regulating red blood cell production. It has three *N*-linked (Asn₂₄, Asn₃₈ and Asn₈₃) and one *O*-linked (Ser₁₂₆) glycan chains, which are often branched and sialylated, to form around 40% of the protein's mass (Figure 6). Removal of its *N*-glycans has little effect on its *in vitro* activity but significantly reduces its *in vivo* activity to less than 10% due to the drug being rapidly cleared by filtration in the kidney [48]. Loss of *in vivo* activity also occurs when EPO is desialylated, with the terminal sialic acid residues removed, in part due to its clearance by carbohydrate receptors in hepatocytes and macrophages. Experiments have also shown that when EPO with variable glycosylation is produced in different mammalian cell lines, different *in vivo* activity was observed [49].

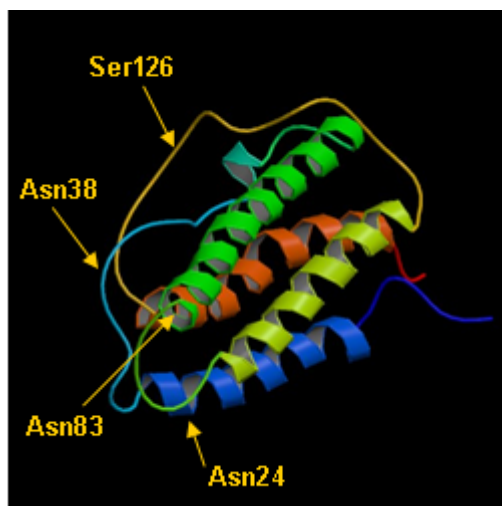


Figure 6: Human EPO, Nuclear magnetic resonance (NMR) spectroscopy minimized average structure (Cheetham *et al.* [50])

IFNs, and more specifically IFN- β and - γ , are another class of cytokines that can be found on the market in its glycosylated form (IFN- α is usually non-glycosylated). IFN- β used in multiple sclerosis and leukaemia therapy, has an *N*-glycan chain at Asn₈₀; on the other hand IFN- γ , used to treat chronic granulomatous disease, can have *N*-glycosylation at Asn₂₅ and Asn₉₇ with varying degree of occupancy [51, 52]. The absence of oligosaccharides in IFN- β results in reduced *in vitro* activity and an increase in protein aggregation [53]. Both glycoprofiles influence the protein's half-life; reflected by the once weekly administering of glycosylated IFN- β compared to multiple doses a week of the non-glycosylated material.

Blood glycoproteins include Factors VIIa, VIII, IXa and XIII, plasminogen activators and antithrombin III. Most of these glycoproteins have one or more *N*-linked glycan chains and some also have *O*-linked sugars. Approved forms of tissue plasminogen activators, despite bearing four potential glycosylation sites, only consist of three modifications at Asn₁₁₇, Asn₁₈₄ and Asn₄₄₈, with Asn₁₈₄ having variable occupancy. Both forms of glycoprotein (with glycosylation at Asn₁₈₄ or without) are biologically active but differ in plasma clearance rates, stressing the importance of monitoring glycosylation during manufacture [54].

Recombinant glucocerebrosidase, a therapeutic, not within the categories above is a glycosylated enzyme containing high mannose chains on four asparagine residues. The molecule is used in enzyme replacement therapy for the treatment of Gaucher disease, a lysosomal storage disorder [55]. The presence of mannose-6-phosphate residues (a trafficking guide for lysosomal enzymes) helps target the enzymes to lysosomes where they can degrade accumulated substrates. Commercial success of glucocerebrosidase has stimulated the development of other lysosomal enzymes for therapeutic treatment.

2.5 Expressing glycoproteins

The glycosylation pattern can differ depending upon the tissue type in which the protein is produced or, in the case of biopharmaceuticals, the expression system used in manufacturing [56]. Bacterial production systems such as *Escherichia coli* (*E. coli*) produce nonglycosylated proteins as an inclusion body that has to be solubilised and refolded *in vitro*. On the other hand yeast (*Saccharomyces cerevisiae* and *Pichia pastoris*) [57-59] and insect [60] expression systems do add oligosaccharide chains but the glycosylation patterns differ not only from one another but also from mammalian ones. Plants are also capable of manufacturing glycoproteins, but regularly add fucose and xylose residues which have been reported to create substructures immunogenic in man [61]. Despite this there have been reports of successful manufacture of plant-derived monoclonal antibodies [62]. The majority of biopharmaceuticals currently use mammalian expression systems producing proteins with glycosylation profiles similar to those found in man (Chinese hamster ovary (CHO), baby hamster kidney (BHK) and NS0 myeloma cells are some of the most widely used). Nevertheless, changes in glycosylation, branching and the degree of sialylation, can arise from variations in cell culture temperature, growth rate, changes in pH and media composition [63]. Hence, expression systems like bacteria, yeast, fungi, insects and plants are currently being explored as alternatives to mammalian cell cultures and the advances and the merits of each one are highlighted in various articles [64-69].

The first transgenically produced protein was approved by the EMEA in 2006 (EMEA/203163/2006). The drug, ATryn®, is a recombinant human antithrombin III anticlotting protein expressed in the milk of transgenic goats by GTC Biotherapeutics. Both ATryn® and antithrombin III derived from human plasma have the same four *N*-linked glycosylation sites (Asn 96, 135, 155, 192) [70]. The glycosylation pattern for the non-human derived product does differ from the plasma one in containing fucose and *N*-acetylgalactosamine residues and a greater proportion of *N*-glycolylneuraminic acid. Other blood factor proteins produced in transgenic animals are currently under development [71].

In general it is essential to choose an appropriate host system for optimal quality and productivity that can, ideally, mimic glycosylation patterns found in humans. By selecting a particular expression system and environment the properties of glycoproteins such as their structure, folding, stability, immunogenicity, uptake, distribution and recognition can also be tailored.

3.0 CHARACTERISATION OF GLYCANS FROM GLYCOPROTEINS

Understandably, the identification of the oligosaccharides structures found on proteins and their function is of importance to both biological research and industrial manufacturing. If, for example, therapeutic glycoproteins are to meet their potential in the treatment of diseases then their characterisation for both quality control (i.e. batch-to-batch consistency) and optimal production is paramount. By-products, either secreted, like glycosidases, or released by dead cells can cause degradation of the

intact product. Thus, during development considerable effort is devoted to monitoring and producing homogeneous recombinant glycoproteins.

Glycan analysis and glycan mapping are the common terminologies used to describe the identification of a population of glycoforms in a protein. Unfortunately, owing to the immense structural diversity of carbohydrates and their complex nature (e.g. sequence, branching, linkages), glycan analysis is particularly challenging, and a broad range of tools have emerged to aid their characterisation, each with its own advantages and disadvantages. Identifying oligosaccharides is further complicated as their detection is difficult, since they do not contain chromophores with suitable absorbance in the ultraviolet region. Additionally, structurally distinct glycans can have the same molecular weight and/or relatively small differences in their physical and chemical properties. Hence, no single methodology is sufficient to achieve a complete picture of the glycosylated protein and, consequently, a combination of techniques is required.

A thorough examination of all the strategies and techniques employed in glycan analysis is beyond the scope of this report, instead the reader is directed towards some general reviews outlining the approaches used for studying protein glycosylation [72-77]. The sections below discuss, in brief, the breakdown of the strategies and some of the methodologies currently used with additional technique based reviews highlighted where possible.

In general, characterisation of the glycoproteins can be broken down into three distinct approaches, as shown in Figure 7:

- i) analysis of the intact glycoproteins
- ii) analysis of glycopeptides
- iii) analysis of the cleaved glycans

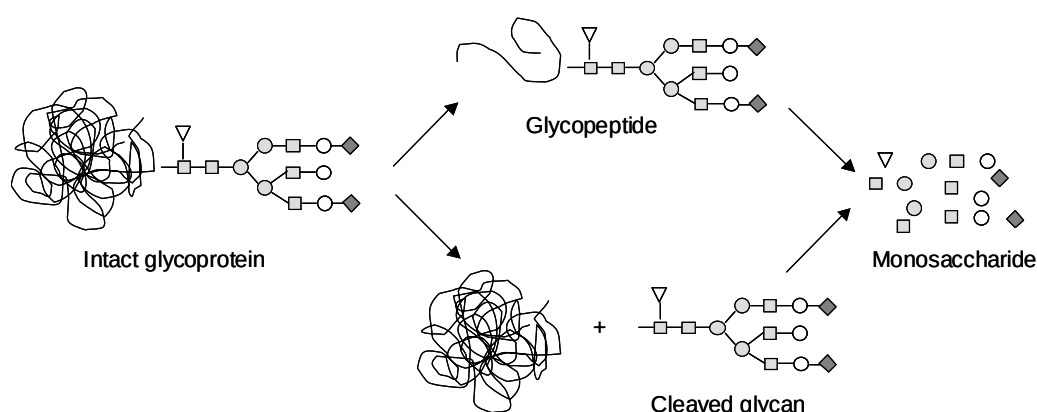


Figure 7: Approaches to analysing glycoproteins

Techniques currently employed in each of these three areas vary significantly and are summarised in Figure 8. A common trend, particularly for the analysis of cleaved glycans and glycopeptides, is to couple separation methods with mass spectroscopy

(MS). General details of MS based methodologies to determine the structure of glycoproteins and glycans can be found in recent reviews [78, 79]. A large portion of investigations carried out on cleaved glycans has been based on fluorophore-labelled systems. Ultimately the tools chosen for the characterisation will depend on:

- i) quantity and purity of material available
- ii) level of information required
- iii) techniques and expertise available to the laboratory

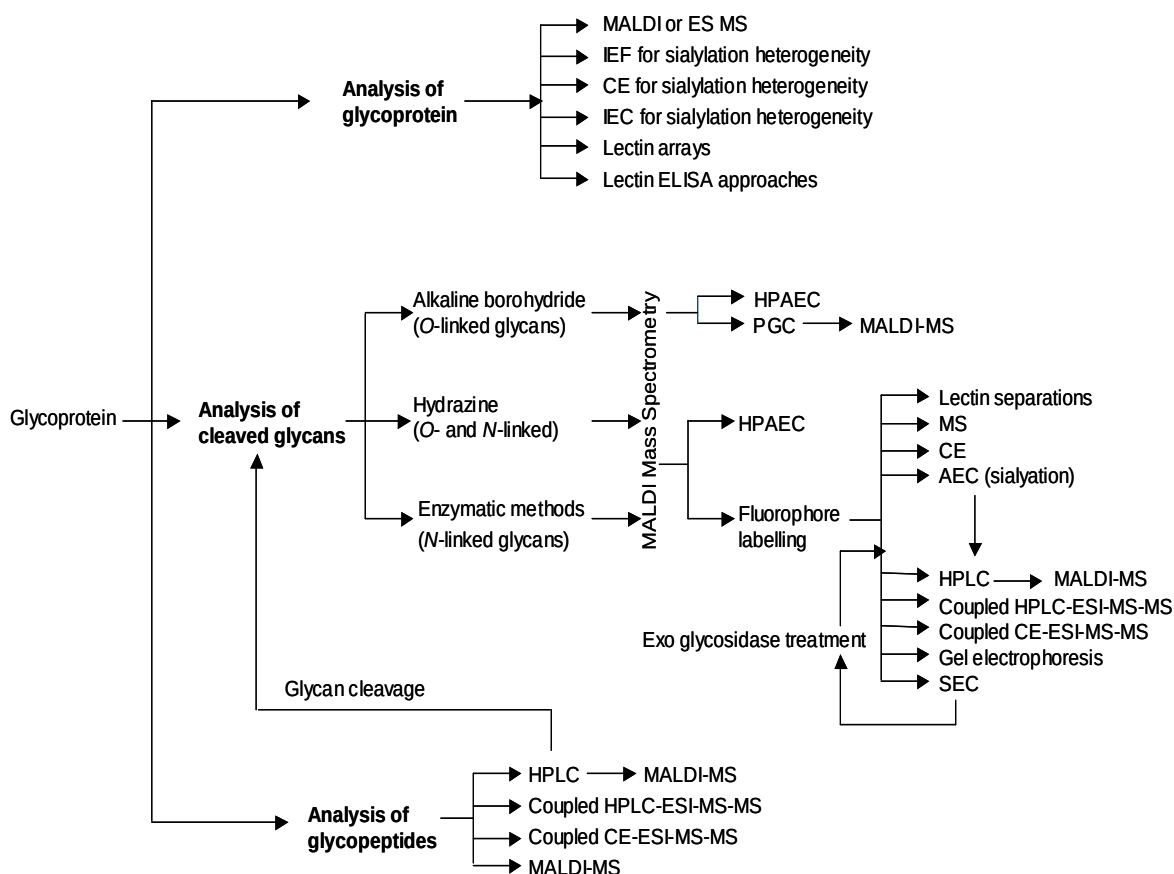


Figure 8: Techniques currently used in glycan analysis

The level of information required can be broken down further into sub-categories such as:

- The site of glycosylation
- Glycosylation linkage (*N*- or *O*-linked)
- Percentage of glycosylation (site occupancy)
- Monosaccharide composition (type and ratio)
- Position and anomeric configuration of glycosidic linkages
- Glycan sequence, including branching
- Sialic acid content (types: *N*-acetyl/*N*-glycolyl)
- Presence of functional groups (phosphate, acetate, sulphate)
- Conformation of carbohydrate moieties

3.1 Analysis of intact glycoproteins

At the intact glycoprotein level, techniques such as gel electrophoresis [80-82], isoelectric focusing, chromatography, lectin affinity chromatography, capillary electrophoresis (CE) [83-85] and MS [79, 86-88] are widely used. In some instances the separation of different glycoforms can be achieved [89, 90], but this is not a widely used technique and it is usually necessary to cleave the biomolecule into smaller fragments constituting of either glycopeptides or glycans. A recent comparison of chromatographic and electrophoretic techniques applied to the characterisation of recombinant protein heterogeneity has been published [91].

Both MS and gel electrophoresis can be used in determining the molecular weight of glycoproteins, with MS emerging over the last decade as a powerful analytical tool for analysing large biomolecules. Lectin affinity chromatography is used to isolate, purify and characterise glycoproteins [92]; immobilised lectins can also be used as bioaffinity probes in an ELISA type format to give an indication of the active glycoforms present in the protein mixture [93-95]. The widespread use of lectin affinity chromatography is currently hindered by the absence of simple, rapid cost-effective procedures for screening different types of lectins. Qualitative and quantitative analysis of glycoproteins can be carried out using capillary electrophoresis, sometimes coupled to MS [84, 85, 96, 97], where its speed of analysis is particularly appealing for applications in process-related environments.

3.2 Analysis of glycopeptides

Glycopeptide analysis, after enzymatic digestion of the glycoprotein, enables characterisation of the carbohydrates with the advantage that the site and the degree of occupancy of glycosylation can be resolved. Procedures applied in amino acid sequence analysis and peptide mapping utilising MS are utilised here. However, glycopeptides from a complex protein digest are not usually directly analysed by MS in part due to the suppression of the glycopeptide signal in the presence of peptides. Suppression of the signal is also observed for glycans containing terminal, negatively charged sialic acids. Thus analysis of glycopeptides involves some form of separation technique such as liquid chromatography [98-100] or CE [101] coupled to MS. Compared to nonglycosylated peptides, glycopeptides have greater complexity and are more likely to be weakly retained on reverse-phase, in addition their ionisation, sensitivity and detection in MS analysis is also lower and, therefore, overall their analysis is more cumbersome and skill-based. Further structural analysis can be carried out by releasing the glycans from the polypeptide chain and characterising the free glycans, described in greater detail below.

3.3 Analysis of cleaved glycans

It is often feasible to look at the state of glycosylation after cleaving the glycans from its protein. This approach enables a more detailed analysis of the glycan structures to be carried out, and a broad range of techniques are available to aid analysis (Figure 8). Because of their complexity, glycan mixtures and glycopeptides alike are frequently separated prior to sequencing and determination of their linkages. Rapid, robust, sensitive and quantitative analytical methods are desired for studying microheterogeneity and, thus, chromatography, CE and MS are often the

complementary techniques used during separation and identification [85, 102-104]. Novotny and Mechref have recently discussed some of the newer hyphenated methodologies based on chromatographic and CE using various combinations of MS [105]. The review by Wuhur et al. looks at some of these options, focusing on recent developments in LC and combinations thereof with MS(/MS) [99].

Ways to illustrate the state of glycosylation in a protein are by describing its:

- i) Monosaccharide composition - involves acid hydrolysis of the glycans to give the relative proportions of sugars present
- ii) Sialic acid profile – gives an indication of the sialic acid content
- iii) Glycan/oligosaccharide profile – cleaved glycans are analysed to give information on the glycan chains (high mannose, hybrid, complex) and on their antennarity
- iv) Galactosylation profile - usually adopted for the analysis of antibodies and refers to the relative proportion of the terminal galactose residues (G0, G1, G2) present in bi-antennary complex type structures

3.3.1 Monosaccharide composition analysis

Quantitative monosaccharide analysis provides precise molar ratios of individual sugars. The general steps in this method are acid hydrolysis of the glycosidic linkages, separation (usually by chromatography) of the resulting monosaccharides followed by their detection and quantification. Chromatography techniques employed include normal and reverse phase, anion and cation exchange, and ligand exchange [106]. Early strategies involved using gas chromatography with MS for quantifying monosaccharides often with permethylation to increase the volatility of the molecules. Simple colorimetric assays, often done on microtitre plates, are also available for determining the total amount of hexose, hexuronic acid and hexosamine residues, but quantifiable information is limited [107]. Problems encountered include the hydrolysis of glycosidic bonds at different rates; sialyl linkages are highly acid labile compared to acid resistant glucosaminyl linkages, as a result there is the possibility that in order to achieve complete hydrolysis liberated monosaccharides can degrade under prolonged or harsh hydrolysis conditions. Thus, to overcome the problem with sialic acid loss a more laborious, two-step procedure is frequently employed, which involves mild acid hydrolysis to remove the sialyl moieties followed by the harsher acidic conditions to release the remaining sugars. In addition, acetylated amino sugars are susceptible to the loss of the acetyl group during hydrolysis rendering this methodology as inefficient.

Analysis by monosaccharide composition provides limited structural information about the glycans (order and branching). In addition, changes in monosaccharide composition may not necessarily indicate major changes in glycan composition that could be of biological significance. For instance some glycoforms may only be present in small amounts and detecting them as changes in monosaccharide composition may be difficult, but these glycoforms could have a disproportionately large influence on bioactivity [108]. The uncertainty of monosaccharide analysis was demonstrated through a study carried out by the old ABRF carbohydrate group, which showed large variations in the monosaccharide composition of a glycosylated sample that was distributed [109].

3.3.2 Sialic acid profile

Sialic acids are found on the termini of *N*- and *O*-glycans habitually linked to a galactose at the α 2-3 or α 2-6 position although α 2-8 linked polysialic acid residues can also be found. The two most common forms present in mammalian cells are *N*-acetylneuraminic acid (Neu5Ac) and its hydroxylated derivative *N*-glycolylneuraminic acid (Neu5Gc). In humans only Neu5Ac is expressed; the functional gene encoding the enzyme to generate Neu5Gc is not present in mankind. In contrast, Neu5Gc is a major component in most mammalian species including Old World Apes [110]. The sialic acid profiling of glycoproteins is of interest to the biotechnology industry since the presence of sialic acid can increase the serum half-life of proteins, with EPO being an excellent example. In some cases the lack of sialic acid also reduces the biological activity of the molecule. The presence of Neu5Gc is also problematic as this particular structure is immunogenic in man. It is therefore important to know about the sialic acid content of a glycoprotein when assaying its function or its efficacy as a therapeutic.

A number of chromatographic methods have been described for sialic acid analysis with weak anion exchange (WAX) and strong anion exchange (SAX) chromatography widely used in biopharmaceuticals quality control for charge analysis of labelled glycans. Alternatively colorimetric assays such as resorcinol [111] and thiobarbituric acid [112] assays can be employed.

3.3.3 Glycan profiling

Analysis of intact glycans released from the protein either enzymatically or chemically provides more detailed information about the various populations of glycans present on the protein. Glycan profiling entails investigating the structure of the carbohydrate chains (i.e. their linkages and isomers) and their antennary (bi-, tri-, tetra-) profiles. Fucosylation and sialylation patterns are also considered here. There is also some form of confirmation of the glycans found, normally by MS, through enzyme digestions or by the use of reference compounds.

Generally some type of fractionation by chromatography or mass spectroscopy is required for separating the glycans prior to investigating the relative molar quantitation of individual structures and their identification. Furthermore, depending upon the technique to be used for glycan profiling the pool of oligosaccharides may need to be derivatised prior to their separation to permit their detection. In brief, glycan/galactosylation profiling involves the following steps:

- release of glycans
- possible derivatisation for detection
- glycan separation
- identification of the various species

In certain cases, particularly when using MS, the steps can be combined into one reducing preparation time and increasing efficiency. The different stages in profiling are discussed further below.

3.3.4 Galactosylation profile

Galactosylation profiling is in fact a form of glycan profiling for complex type biantennary *N*-glycans. The glycan content of monoclonal antibodies is regularly described through terminal galactose units terminology: G0 (agalactosylated), G1 (monogalactosylated) and G2 (digalactosylated). These galactosylated glycans are of particular interest as the distribution of these glycoforms affects *in vivo* Fc effector functions.

3.4 Practical aspects of profiling glycans

This section outlines the practical aspects of analysing protein glycosylation: the emphasis is on the analysis of cleaved glycans as this is the subject of this report. The cleavage, separation and identification of glycans will be discussed.

3.4.1 Cleavage of glycans

Glycans can either be chemically or enzymatically released from their respective proteins, with cleavage of *N*-glycans using *endo*-glycosidases (Endo-H and Endo-F) and Peptide-*N*-glycosidase F (PNGaseF) the most widely employed methods due to their broad specificity and their wide commercial availability. PNGase F, which cleaves the Asn side chain amide to release the sugar amine, is the most widely used enzyme. However, the enzyme does not release all types of *N*-glycans such as those from plant or insects, which contain α 1-3 linked core fucose residue, and it does not work on smaller glycopeptides such as those obtained by tryptic digests. The alternative is PNGase, A which has broader substrate specificity, but it is more expensive and less widely available. Endo H cleaves high mannose and hybrid structures by hydrolysing the bond between chitobiose (GlcNAcGlcNAc) in the core pentasaccharide region; consequently information about fucosylation is lost. In contrast, no enzymes with broad specificity for the release of *O*-linked glycans exist and less research has been done using this approach. The highly specific *O*-Glycanase (*endo*- α -*N*-acetylgalactosaminidase) only cleaves the short, neutral *O*-glycan chain, Gal- β 1,3-GalNAc between the GalNAc linkage to Ser/Thr [113]. No cleavage occurs if the disaccharide is modified, for example by sialylation, sulphation or elongation. Thus, since there are at least eight different types of *O*-glycan core linked to serine and threonine, chemical release of these sugars over enzymatic is more common.

Chemical release of both *N*- and *O*-linked oligosaccharides can be achieved by hydrazinolysis, a method that effects complete release and yields unreduced glycans. The conditions for release can even be optimised so that there is selective removal of *O*-glycans (mild hydrazinolysis at 60°C) followed by release of *N*-glycans (hydrazinolysis at 95°C) [114]. A drawback with this method is that although it leaves the glycans intact the protein component is destroyed and often, particularly for *O*-glycans, “peeling” of the terminal sugar moiety when it is substituted in the 3-position can occur [115]. *N*-Acetyl groups on the sugars can also be hydrolysed and an additional re-*N*-acetylation step is needed. Unfortunately the bigger problem is that commercial availability of anhydrous hydrazine is very limited due to its highly explosive nature, and as a consequence other methods of releasing glycans (particularly *O*-glycans) need to be investigated. One such chemical method is alkaline β -elimination in the presence of reducing agents, widely used for *O*-glycans and is as established as hydrazinolysis for *N*-glycans. However, this method yields

alditols without a reducing end preventing their direct derivatisation to fluorophores [116]. A recently modified β -elimination procedure using ammonia, which leaves the reducing ends intact, is now becoming more common [117].

In conclusion, the limited availability of hydrazine for hydrazinolysis has led to most groups switching to PNGase F for the routine release of *N*-glycans. In comparison, routine procedures to release intact *O*-linked glycans with free reducing termini are not as common, thus hindering *O*-linked glycan analysis.

3.4.2 Separation of glycans

The technique to be used to fractionate the heterogeneous glycan mixture prior to structural analysis depends on the materials (derivatised or non-derivatised) and the availability of different chromatographic instruments. For example, if a chromatography based method is to be employed, then generally the carbohydrate mixture will be chemically derivatised to aid detection. An exception to this is when a high-pH anion-exchange chromatography with pulsed amperometric or electrochemical detection (HPAEC-PAD or -PED) system is utilised [118, 119]. On the other hand, if the technique is MS, then it is not always necessary to derivatise glycans prior to their analysis.

The type of derivatisation carried out will depend on the method that is to be used to detect the glycans: radioactive, colorimetric or fluorescent. Procedures for radioactive detection include labelling by reduction with sodium borotritide of aldehyde functionality found on either the reducing end of sugars or the sialic acid side chain after mild periodate oxidation. Derivatisation for colorimetric and fluorescent approaches is typically achieved through the chemical modification of the aldehyde functionality located at the free, reducing end of sugar moieties. For example, a common practice when using HPLC is to fluorescently tag sugars, *via* a reductive amination reaction, to enhance the sensitivity of glycan detection to the pico- and femtomole range [120]. The advantages and disadvantages associated in this approach have been summarised by Wang *et al.* [121]. Fluorophore labelling and post-labelling cleanup kits are available to assist reproducibility and turnover.

Permethylation of the hydroxyl groups found on glycans with sodium hydroxide and methyl iodide is another derivatisation method: mass spectrometrists frequently use this approach. The presence of methyl groups (and fluorophores) increase the ionisation properties of these molecules compared to the non-derivatised ones and hence improves detection by MS [122]. Permethylation also leads to a more informative fragmentation pattern aiding glycan structure determination. In addition, esterification of the carboxyl group on sialic acids during methylation neutralises the sugar and reduces the loss of this moiety during ionisation. Introducing a label at the reducing end is also a versatile way to exploit detailed analysis using a series of *exo*-glycosidases: the attachment of a fluorophore allows glycans to be monitored *via* a chromatographic system as each terminal sugar residue is cleaved, and fractions can be recovered for further digestions.

Other separation techniques used in industry, apart from chromatography and MS, include gel filtration, gel electrophoresis [123], CE [85, 124, 125] and lectin-affinity chromatography. The high-resolution capabilities of CE, using fluorophore labels if required, means that it is becoming a widely used separation in industry.

Oligosaccharide profiles obtained during separation, from techniques like HPLC and CE, can be used for routine comparative studies and to acquire preliminary information about the number, relative quantities and types of oligosaccharides present in the glycan mixture.

3.4.3 Identification of glycans

Full structural elucidation of the separated glycans (including determination of the anomeric configurations of the glycosidic linkages) can reliably be resolved using NMR spectroscopy but, a sufficient quantity of pure individual material is required in order to perform this technique (typical at least 100 µg). Alternatives to NMR spectroscopy include identifying glycans through their molecular weight by MS [87, 126-130]. When fragments of the glycan ions are analysed in MS/MS experiments, information about branching and linkages can be obtained. The main MS methods presently used are the soft ionisation techniques Matrix Assisted Laser Desorption Ionisation Time-of-flight (MALDI-TOF) and Electrospray ionisation (ESI-TOF). Sequential *exo*-glycosidase treatments enable the anomeric configuration of specific terminal sugars to be deduced, but the number of enzymes available is limited and the procedure is labourious. Comparison of analytical data of the unknown glycans with literature and known glycan standards can also be carried out. However, a drawback with using “well-characterised” glycan standards is the limited number of pure oligosaccharides available. Employing mass spectroscopic-based techniques is particularly convenient as the separation and identification of glycans through mass/charge (*m/z*) ratios can be achieved in one step in a rapid, high throughput manner, with derivatisation of the sugars optional. One main drawback/disadvantage using MS alone for glycan mapping is that it may not be quantitative due to differential efficiency in ionisation for different types of oligosaccharides, indeed, more work is still needed to show otherwise.

Table 2 summarises the different analytical techniques, and the specific methods within them, that can be employed to separate and analyse glycans that have been cleaved from their glycoproteins.

Table 2: Techniques employed to analyse cleaved glycans

Analytical Technique	Specific Method
Electrophoresis	Polyacrylamide gel electrophoresis (PAGE) Capillary (zone) electrophoresis (C(Z)E) Capillary Electro chromatography (CEC)
Chromatography	Anion exchange (AEC) Porous graphitised carbon (PGC) HPAEC-PAD Lectin Monolithic columns Normal phase/amide (NP) Reverse phase (RP) Size exclusion (SEC)

MS	CE-ESI-MS(/MS) ESI-MS(/MS) ESI-(Q)-TOF MS HPLC-ESI-MS(/MS) MALDI-TOF-(TOF)-MS
Other	Isoelectric focusing (IEF)

4.0 INTER-LABORATORY STUDY

Unlike the biosynthesis of proteins, which is defined by the gene introduced, protein glycosylation is variable and dependant on the host cell. It is also sensitive to changes in growth conditions of the cell and environmental stresses. As discussed in the chapters above, variations in glycosylation can influence the biological activity of the protein including its *in vivo* half-life, stability and modulatory functions. Hence, monitoring protein glycosylation for reproducibility is an important quality control measure for many drugs.

The complexity and flexibility of glycan analysis puts great demands on the numerous analytical tools available for their characterisation. There is the added problem that multiple techniques are usually required, and so there is a genuine need to assess the comparability of the different glycan analysis methodologies, with the aim of harmonising the field to support regulatory approval. Correlation of the key structural features required for biological activity with the techniques utilised in analysis will provide an understanding of the variability issues that can arise when comparing multi-platform methods. This projects aims to address some of these issues with an inter-laboratory study.

Analysis of glycosylation *via* cleavage of the glycan chains is the more generic approach used both in product characterisation and quality control; therefore, this is the focus of this study. Furthermore, this study deals only with *N*-linked glycan chains, as these are the ones that have been, on the whole, studied the most with respect to their influence on the biological activity of therapeutic materials.

4.1 Study design

The study consisted of two components, a questionnaire and a laboratory/analytical-phase study. The questionnaire was designed to find out about the methods employed by participants in the laboratory study and to aid data analysis. The laboratory study involved participants around the UK analysing mixtures of *N*-glycan chains using their routine methodologies; the amount of information obtained about the glycan structures, and its variation in data reported by these methods and their comparability were to be evaluated.

For the inter-laboratory study *N*-glycan chains released from different glycoproteins were distributed to participants for glycan analysis. Providing released *N*-glycans enabled the study to focus solely on the variations observed during glycan analysis and interpretation, and eliminate inaccuracies arising during release and isolation of the *N*-glycans. This step is particularly important, as it is known that *N*-glycanases have lot-to-lot variations in enzyme activity, *exo*-glycosidase contamination and variations in its formulation that could interfere with subsequent workups. PNGase F was the enzyme selected for cleavage of the *N*-glycans.

Four samples of *N*-glycans was considered a suitable number to investigate the range of glycosylation patterns found in nature, without being too time consuming for the laboratories volunteering to take part in the study. Participants were informed that their glycan samples were of the *N*-linked type with a free reducing terminus to enable derivatisation if they desired. The identity, however, of the glycoproteins that the *N*-

glycans were derived from was not revealed until after the study was complete; this was to ensure unbiased results.

Participants were also provided with (i) a standardised spreadsheet to aid reporting of glycan structures, (ii) the questionnaire, (iii) an instruction guide and (iv) a letter outlining the aims of the study. Samples were distributed over the summer of 2006 and individual laboratories were given a maximum of three months to run the samples and return the results of their findings. It was stressed to the participants that the results obtained would remain anonymous with only the project coordinators, NPL and NIBSC, having access to the original data set.

4.2 Selection of glycoproteins

The four glycoproteins selected were: human α -1 acid glycoprotein (H α 1), bovine α -1 acid glycoprotein (B α 1), bovine pancreatic ribonuclease B (RNase B) and human immunoglobulin G (IgG). These glycoproteins have glycosylation patterns typical of those present on therapeutic proteins, and the rationale behind selecting these glycoproteins is discussed below:

- α -1 acid glycoprotein is a heavily glycosylated protein containing complex bi-, tri- and tetra-antennary *N*-linked glycans that are highly sialylated. Depending on the source of the glycoprotein, such as human and bovine used in this study, different complex sialylated structures are found, including non-human sialic acids such as Neu5Gc. Therefore, H α 1 and B α 1 were selected to see if these differences were detectable during routine glycan analysis, particularly as they can have therapeutic repercussions.
- RNase B was selected as it contains mainly neutral *N*-glycans of the high-mannose type. Glycan samples from this source were selected to enable a comparison of data that did not necessitate analysis in the presence of charged sugars.
- IgG was chosen as a glycan source as antibodies are the most popular commercial therapeutic glycoproteins. The molecule contains mainly highly fucosylated complex bi-antennary glycan chains with varying degrees of galactosylation, with some minor structures afucosylated and/or with bisecting GlcNAc residues. IgG, in addition, possesses minor amounts of sialylation and it allowed comparison between its oligosaccharide and sialic acid profiles and those of glycoproteins exhibiting large amounts of terminal sialic acid.

4.3 Samples for analysis

Samples of *N*-glycans chains (A, B, C and D), prepared by PNGase F deglycosylation of the four selected glycoproteins were purified and aliquoted into plastic vials. Each contained approximately 25 μ g of underivatised sample. Table 3 reveals the identity of the four *N*-glycan samples A, B, C and D. 25 μ g of material was considered sufficient for distribution as it is typical of the amount normally evaluated whilst, at the same time, it allowed analysis by more than one technique to be carried if desired. Also distributed was 25 μ g of underivatised dextran ladder, a commonly used reference material, which participants had the option of using for method calibration.

Table 3: Identity of the samples distributed to participants

Sample	Source of glycoprotein
A	H α 1
B	B α 1
C	Bovine pancreatic RNase B
D	Human IgG

GyroVap dried samples were shipped at room temperature in 1.5 mL screw capped micro centrifuge tubes and stored at -20°C until analysis. Preliminary studies were carried out to ensure that the distribution of the samples at room temperature could be achieved without degradation.

4.4 Level of information required

This study focussed on the oligosaccharide and sialic acid profiles of released *N*-glycans; analysis of the monosaccharide composition of the *N*-glycan mixtures was not requested. Information about the identity and relative percentages of as many of the structures present in the samples A, B, C and D was to be realised by the participants (detailed linkage analysis was optional). For glycans A and D, the participants were asked to provide additional information about its sialic acid content (e.g. percentage of sialic acid present) as well the required general information about their identity and relative ratios.

Participants were asked to analyse the samples using the laboratory's routine methodology. At no stage was any preference shown as to what technique or protocol the organisation should follow, though it was envisaged that a good portion of the laboratories would perform some form of separation (chromatographic or electrophoretic) followed by verification of the peaks using on-line or off-line MS. Participants were asked to report their findings on a standardised Excel data sheet and also, where possible, provide copies of their raw data.

4.5 Glycan analysis questionnaire

A copy of the questionnaire can be found in Appendix 11.1. It consisted of three parts: i) glycan separation, ii) glycan identification and iii) the use of quality laboratory systems. The questionnaire was designed to reflect the range of techniques utilised to analyse the glycans. For instance, we wanted to capture whether laboratories preferred to use a less common method, a combination of a few methods or even something not listed in the questionnaire.

4.6 Data analysis spreadsheet

To facilitate the inter-laboratory comparison, the participating organisations were requested to document their data using a standardised reporting spreadsheet and by

expressing the different glycans observed as relative percentages. The glycan data spreadsheet was provided in Excel format and a copy can be found in Appendix 11.2.

The spreadsheet was divided into five sections for reporting: high mannose chains, hybrid chains, complex bi-, tri- and tetra-antennary chains. Each section listed the major *N*-glycans that could occur within it, but given the vast number of possible structures some of the rarer ones were omitted. Nonetheless there was opportunity at the bottom of the spreadsheet to include any structures that were not listed. The spreadsheet also tried to take into account that laboratories may identify and quantify structures in different formats depending on the technology they employed. For example, depending on the technique, the individual isomers of the high mannose glycan Man₇GlcNAc₂ can be separated and identified individually, alternatively they can be recognised collectively as one group. To take this into account two columns were provided next to the listed glycans to record their percentages; one for the individual glycans and the other for glycans observed as a group. A small sample of the spreadsheet is illustrated in Table 4.

Table 4: Example of a section of the spreadsheet

Complez bi- antennary chains	% of glycans found	
NGA2 or G0		
NA1(a) or G1(a)		
NA1(b) or G1(b)		
NA2 or G2		
NGA2F or G0f		
NA1F(a) or G1f(a)		
NA1F(b) or G1f(b)		
NA2F or G2f		
A1(a) or G1S1(a)		
A1(b) or G1S1(b)		
A1(c) or G2S1(a)		
A1(d) or G2S1(b)		
A2 or G2S2		
A2F or G2fS2		

The spreadsheet was developed to facilitate participants matching the structures they found in the glycan samples to the abbreviated glycan list on the data sheet, by integrating into the spreadsheet a page of pictorial representations of the *N*-glycan structures. The figures were split into the same five sections for as for reporting. The figures were standardised to favour no one particular group or style, as shown in Figure 9. The full sets of *N*-glycan drawings are located in Appendix 11.3.

 GN-M M-GN-GN GN-M NGA2 G0	 G-GN-M M-GN-GN GN-M NA1(a) G1(a)	 GN-M M-GN-GN G-GN-M NA1(b) G1(b)	 G-GN-M M-GN-GN G-GN-M NA2 G2
 GN-M M-GN-GN GN-M NGA2F G0f	 G-GN-M M-GN-GN GN-M NA1F(a) G1f(a)	 G-GN-M M-GN-GN GN-M NA1F(b) G1f(b)	 G-GN-M M-GN-GN G-GN-M NA2F G2f
 SA-G-GN-M M-GN-GN GN-M A1(a) G1S1(a)	 GN-M M-GN-GN SA-G-GN-M A1(b) G1S1(b)	 SA-G-GN-M M-GN-GN SA-G-GN-M A2 G2S2	 SA-G-GN-M M-GN-GN SA-G-GN-M A2F G2fS2

Figure 9: Sample of the figures used in the data spreadsheet

Glycans represented in the spreadsheet were written in an abbreviated letter format frequently encountered in literature. Generally, glycans from which the sialic acid is absent was designated by the prefix NA (asialoglycans), while the lack of both sialic acid and galactose was shown as NAG (asialo-agalactoglycans). In some cases, such as the complex bi-antennary chains, two sets of configurations were used; this is because there is more than one popular way to depict these glycans. For example, a bi-antennary structure consisting of a fully galactosylated structure but no sialic acid residues would be written as either NA2 or G2. The same structure but fully sialylated would be A2 or G2S2, as illustrated in Table 4.

Participants were encouraged to use this reporting format only to aid the comparison of data between different laboratories. It is hoped that the Excel spreadsheet will resolve the problem of inconsistent glycan nomenclature and reporting of data. It should be noted that the study organisers have no preference for one particular nomenclature system over another.

5.0 MATERIALS AND METHODS

5.1 Materials

Glycoproteins human α -1 acid glycoprotein (G9885; Ha1), bovine α -1 acid glycoprotein (G3643; Ha1), bovine pancreatic ribonuclease B (R7884; RNase B) and human antibody (I4506; hIgG) were all purchased from Sigma-Aldrich and used without further purification. PNGase F from *Flavobacterium meningosepticum* (P0704L) was obtained from New England BioLabs as a kit containing: 10X denaturing solution (5% SDS and 400 mM dithiothreitol), 10X G7 reaction buffer (500 mM Na₂HPO₄ pH 7.5), 10% detergent solution (NP-40). C18 (Oasis HLB) mini cartridges were from Waters. All other reagents were purchased as reagent grade materials from Sigma-Aldrich and unless otherwise stated were used without further purification. Water was of analytical grade (ELGA or milli-Q-water).

5.2 Deglycosylation with PNGase F

Cleavage of the N-glycans was carried out using the manufacturer's (New England BioLabs) protocol with some modification. Each glycoprotein (5 mg) was dissolved separately in water (500 μ L) in a 2 mL micro centrifuge tube, and then denatured with

10 X denaturing solution (55 μ L) at 100 °C (boiling water bath or heat block) for 10 minutes. To the cooled solution was added the following: 60 μ L of 10X G7 reaction buffer, 60 μ L of 10X detergent solution and 10 μ L of PNGase F enzyme solution (5000 units). The samples were incubated at 37°C for a period of 24 h and the reactions stopped either by purifying the material (Section 5.3) or by freezing at –20 °C, for purification at a later date.

5.3 N-Glycan purification

5.3.1 Precipitation of protein and purification of released N-glycans

Protein from the reaction with PNGase F was precipitated by the addition of water (150 μ L) and 1 M acetic acid (150 μ L). After cooling in the fridge (4-8 °C) for approximately 10-15 minutes, the solution was vortexed and centrifuged (5000g, 2 min) and the supernatant transferred onto a mini C18 cartridge (one per each deglycosylation reaction) for removal of the remaining soluble protein/peptides.

Prior to use, each mini C18 cartridge (30 mg Oasis HLB, Waters) was conditioned with ethanol (0.2 mL) and equilibrated with 50mM acetic acid/5% ethanol solution (1 mL). To the conditioned cartridge was added the supernatant from the PNGase F digest. The supernatant was allowed to pass through at atmospheric pressure into a collection vessel (10 mL disposable polypropylene test tube).

The precipitate was washed 3 x (1x 200 μ L, 2 x 300 μ L; vortex and spin down) with 50 mM acetic acid/5 % ethanol solution, and the washes passed through the same C18 cartridge to elute the N-glycans. Proteins and peptides were retained on the cartridge, which was discarded after use. The combined flow through was lyophilised and stored at –20 °C prior to further purification by Amide-80 normal-phase HPLC.

5.3.2 Additional purification of N-glycans by normal-phase HPLC

The N-glycans were purified further by HPLC to remove any remaining soluble peptides and salts using a TSK Amide 80 column (5 μ ; 4.6 x 250 mm; Anachem) [131]. Hydrolysed chitin oligomers were used as quality control materials for assessing the column efficiency. Lyophilised N-glycans from the C18 cartridge were taken up in solvent B (100 μ L; 70 μ L first and then 30 μ L wash) and the entire amount injected into the HPLC. The mobile phase was aqueous acetonitrile (ACN) in 5mM TFA/(10mM NH₄OAc pH 4.2), final pH 2.5; Solvent A = 80 % ACN; Solvent B = 20% ACN. Flow rate was 1.0 mL/min; UV detection was at 228 nm and the chromatography performed at room temperature. Elution gradient was: t₀-5 = 30 % B, t₇-10 = 100 % B, t₁₂-20 = 30 % B. Fractions between 8 and 11 minutes containing the N-glycans were collected and lyophilised.

HPLC was performed on an inert Gilson binary gradient system (Anachem) fitted with a model 118 UV detector and a model 122 fluorescence detector. Operation and data acquisition were handled by the Unipoint computer software package (Anachem).

5.4 Calculating *N*-glycan content

The concentrated *N*-glycans samples were reconstituted into water (500 μ L) and the hexose content determined by orcinol staining [132]. Briefly, the *N*-glycans were spotted onto a thin layer chromatography (TLC) plate alongside a range of galactose concentrations (0.6-0.05 mg/mL) for a calibration curve. The sample spots were then sprayed with an orcinol solution (900 mg of orcinol, 25 mL of water, 375 mL of ethanol, 45 mL of 18 M H₂SO₄) and the plate charred at 110 °C for 1 minute in an oven or until the spots turned violet indicating the presence of hexose. Densitometry was performed on the purple spots after cooling the TLC plate and the hexose content determined from a galactose standard curve. From the hexose content, the total *N*-glycan weight (including sialic acid and *N*-acetylglucosamine residues) can be estimated by multiplying the hexose content with the following factors: x3 for H α 1 and B α 1; x 1.5 for RNaseB; x 2 for hIgG.

The purified glycans samples A, B, C and D, were divided into aliquots each containing 25 μ g of oligosaccharides per 30 μ L in 1.5 mL micro centrifuge tubes, dried down by GyroVap (VA Howe & Co Ltd., UK) and labelled ready for dispatch. The samples were stored at –20 °C.

5.5 Stability studies of the cleaved *N*-glycans

Studies on the stability of the cleaved *N*-glycans were performed by shipping out two lots each (25 μ g total oligosaccharide) of dried RNaseB, IgG, Transferrin (Tf), Fetuin (Fet), and H α 1 *N*-glycan samples to the USA at room temperature using ordinary airmail post. The samples were returned and analysed at NIBSC for comparison with three lots of the same batch of material, which had been stored at –20 °C. Prior to evaluation by HPLC using a TSK Amide 80 column, the *N*-glycans were labelled with a fluorophore [131].

No difference was observed in the *N*-glycan HPLC profiles between the posted batches and those stored at –20 °C. Batches of *N*-glycans analysed included structures, which ranged from neutral high-mannose type (RNase B), weakly acidic complex type (IgG) and highly acidic bi- (Tf), tri- (Fet) and tetra-antennary complex types (H α 1). Typical HPLC profiles for these *N*-glycans can be found in the article by Yuen *et al.* [131]. This experiment showed that the glycans samples were stable at room temperature for at least two weeks, and it also indicated that the quantity dispensed in each of the micro centrifuge tubes was consistent.

6.0 RESULTS AND DISCUSSION

A total of eleven laboratories around the UK took part in the comparison study; these included UK-based biopharmaceutical manufacturers, academics and analytical contract laboratories. Samples were distributed to the majority of participants in July 2006 and approximately three months given for the return of results. The following section describes the outcome of the questionnaire and the inter-laboratory study and is split into respective sub-sections.

6.1 Results from questionnaire

Responses from the questionnaire were collated into an excel spreadsheet and the results are discussed below.

6.1.1 Glycan separation

Q1. What do you separate/analyse?

This question gave a total of twenty responses, indicating that many participants analysed both derivatised and underivatised glycans (Figure 10) (55 % analysed both types of glycans). The use of underivatised and fluorophore-labelled glycans proved equally popular amongst participants. In some cases (10 % of the total) analysis was carried out after desialylation of the oligosaccharides. The category “other” consists of permethylated glycans. This question shows that the analysis of underivatised sugars is popular amongst participants, particularly when using MS (data not shown).

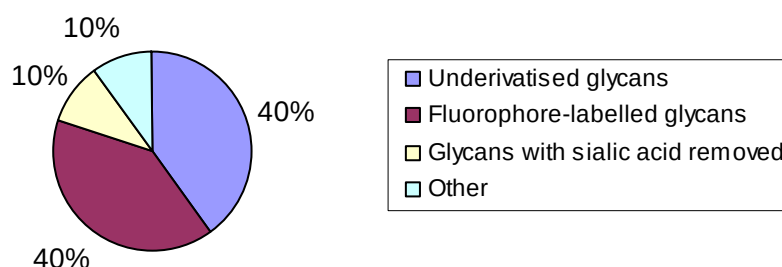


Figure 10: Type of material used for analysing glycans

Q2. How do you separate/analyse the glycans?

Results from this question, shown in Figure 11, highlight that the majority of analyses are carried out using chromatographic and mass spectroscopic techniques. MALDI and ESI (82 % and 27 % of participants, respectively) were the two frequently used techniques for MS analysis, whilst normal-phase HPLC and anion-exchange (64 % and 45 % of participants, respectively) were the popular methods for chromatography. Between the eleven laboratories a total of thirty-two methods were used, averaging out to just under three per organisation. The answers showed the previously popular technique HPAEC-PAD is no longer as widely used as in the past. Less common techniques were hydrophobic interaction chromatography (HILIC), fluorophore assisted capillary electrophoresis (FACE) and size exclusion chromatography (SEC).

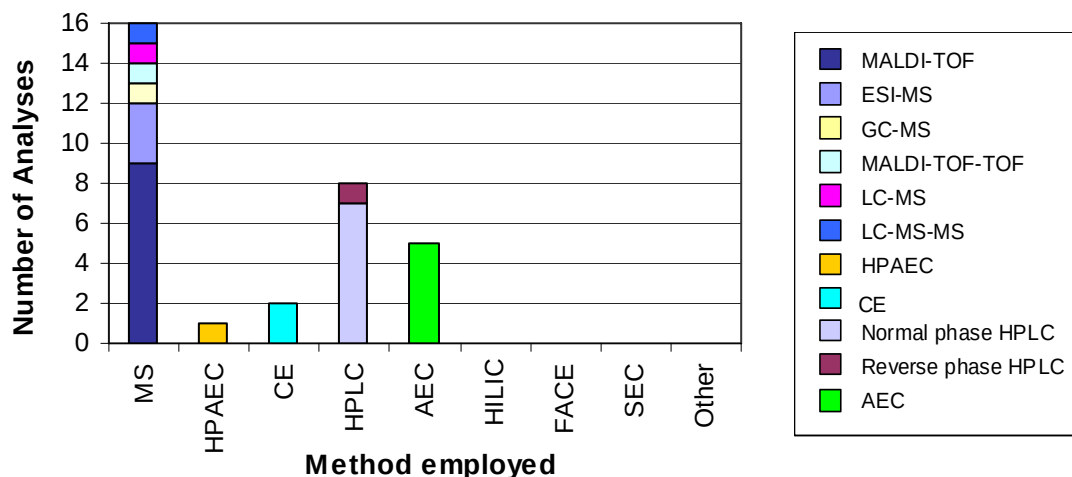


Figure 11: Techniques used by the participants to analyse *N*-glycans

Q3. If you use MS, do you use a matrix?

Participants stated that DHB was the reagent of choice when MALDI-TOF-based MS techniques were utilised, although in some case more than one matrix was investigated.

Q4. If you use fluorescent labelling, which fluorophore do you use?

This question produced a total of nine replies, with some laboratories using more than one label depending on the technique they used; nonetheless, 72 % (eight laboratories) of the organisations stated that they used fluorescent labelling. Of the labels employed 2-AA and 2-AB were the two favoured ones (Figure 12) with 33 % and 45 %, respectively. Understandably the label APTS was used for labelling *N*-glycans separated by CE, this label can also be used for fluorophore-assisted carbohydrate electrophoresis (FACE) but in this study this technique was not investigated.

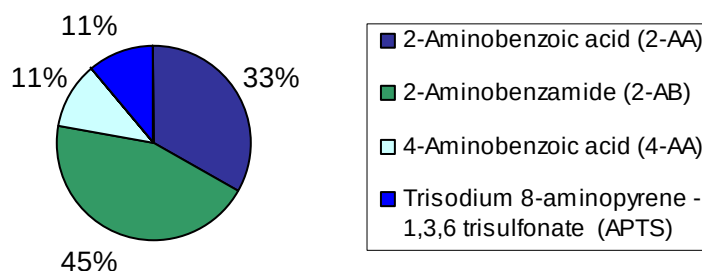


Figure 12: Type of fluorophore used to label the glycans

Q5. For fluorescent labelling, do you use a commercial kit?

Participants that used fluorophore labelling were asked if they used any commercial kits and, six out of the eight laboratories replied positively indicating that they did and that their supplier was Ludger Ltd. In one case a Beckman Coulter kit was also used.

The popularity of 2-AA and 2-AB fluorescent tags could be explained by their exploitation through commercial kits.

Q6. If you use HPLC, how do you detect peaks from the column?

Detection using a fluorometer was the predominant method used by the participants who exercised HPLC as a glycan mapping technique (Table 5). From this survey, PAD, which was heavily used in the nineties, has now been replaced by the more sensitive fluorescence based detection for routine analysis.

Table 5: Detection system used with the HPLC

Detection	Responses
PAD	0
PED	0
UV/Vis absorbance	1
Fluorescence	7
LIF	1

6.1.2 Glycan identification

Q7. How do you assess instrument performance?

The results from this question were clearly dependant on the technique used by the organisation. The majority of participants used both a dextran ladder and commercial/in-house reference glycan mixtures (69 % of the total responses) as a way of monitoring their measurements (Figure 13): every chromatography-based testing performed used these two measurement tests. For mass spectroscopic methods a few laboratories mentioned that they used peptide mixtures for calibrating their instrument. Some stated that in addition to reference glycan mixtures or a dextran ladder they used other reference standards, such as individual glycans.

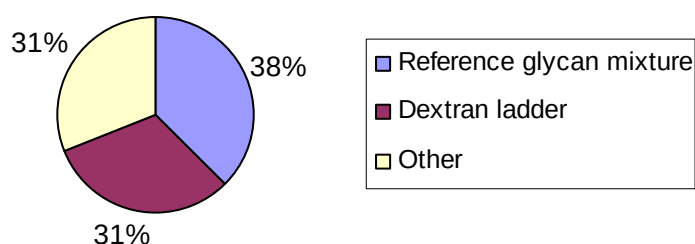


Figure 13: Methods used by participants to qualify instrument performance

Q8. How do you identify peaks?

Figure 14 shows that more than one method was used per laboratory to aid the identity of the glycans, with determination of molecular weight by MS the most frequently used technique (82 % of participants). Identification of oligosaccharides from chromatographic profiles displayed the greatest selection of methods, including

on-line or off-line MS, *exo*-glycosidase digestions and the comparison of retention times with reference materials and/or glucose units (GU) using a dextran ladder. Although *exo*-glycosidase digestions strategies were employed, this approach was not as popular as MS. The category of ‘other’ mainly consisted of identification through databases.

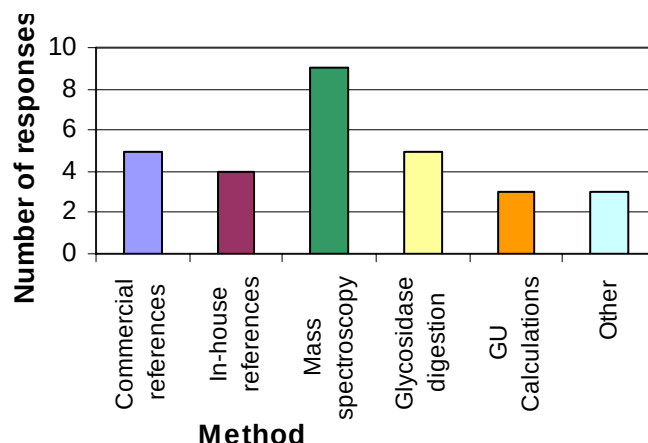


Figure 14: Method used to identify the glycans

Q9. How do you quantify peaks?

A large portion of the people surveyed use integration of a trace (64 %) to quantify the peak, in other words the laboratories working with chromatography used integration to report relative percentages of glycans. MS users preferred to look at relative abundance/peak height for their peaks.

Q10. Do you correct quantified peaks for differences in response factor?

All participants stated that they did not correct peaks for response factor.

Q11. What values do you report?

The same laboratories that quantified their peaks by integration of peak area reported their data as a percentage of molar ratios of glycan components. The mass spectrometrists reported their measurements as percentages using relative abundances.

6.1.3 Quality aspects

Q12. Do you routinely qualify your methods?

The number of laboratories that routinely qualify their methods was 73 %; one group (9%) did not respond to this question.

Q13. Do you routinely validate your methods?

Compared to question 12 only 54 % of the participant said that they did validate their methods, the other 36 % did not and one laboratory did not reply.

Q14a. Do you perform performance qualification on your instrumentation?

As illustrated in Figure 15 this question produced a range of responses. Most organisations (91 %) performed some form of check on their instrument; however, the responses could not be related to the different sectors - biopharmaceutical manufacturers, academics or contract laboratories. The stage in which tests were done most frequently was annually.

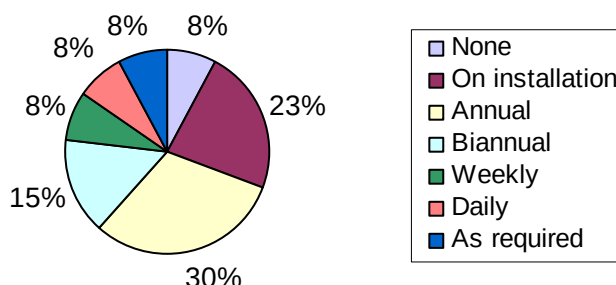


Figure 15: Frequency of performance qualification undertaken on instrument

Q14b. If yes for Q14a, who performs the qualification on your instrumentation?

From the laboratories that carried out performance qualification on their instruments, 15 % used staff, 46 % manufacturer's personnel and 39 % a qualified operator.

Q15. Do you qualify your reagents?

Just under half (45 %) of people stated that they qualify their reagents.

Q16. Does your laboratory operate under one of the following quality systems?

Responses from this question demonstrate that a range of quality systems are utilised by organisation with no quality system being the largest category (Figure 16). Four other systems were cited: cGMP, cGLP, ISO 17025 and ISO 9000; all in equal proportions.

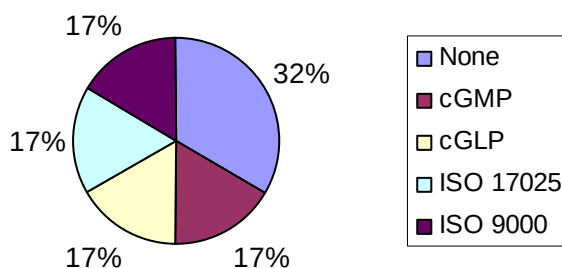


Figure 16: Quality system laboratory under operation

6.2 Results from inter-laboratory study

This section looks at the results of the data received by the eleven participating laboratories for the analysis of the four samples A, B, C, and D. The *N*-glycan structures reported by organisations were collated into an Excel spreadsheet; a separate worksheet was used for each sample. The values for the relative percentages for each glycan found were summed according to category for data presentation and the results are discussed below.

For anonymity the organisations were given a coded letter, which is used throughout this section. Where data was not presented as relative percentages or where there was insufficient information no letter was assigned. In instances where this occurred, data was collated and represented as the total number of glycans found for that particular technique. In some cases laboratories reported data from two separate techniques and where this is the situation a separate letter was assigned. The letters were assigned in a non-alphabetical manner and in no particular order.

6.2.1 Sialic acid profile

6.2.1.1 Overall extent of sialylation

Although participants were originally asked to investigate the total percentage of sialic acid containing glycans in samples A and D only, the data received by each participant (Labs A to J) provided information on all four samples, indicating that laboratories are aware of the importance of sialic acid determination. Each participant showed similar trends in the total amount of sialic acid present on glycans, which is illustrated in

Figure 17, these were:

- Samples A and B were estimated to contain nearly 100 % sialylated glycans
- Sample C had virtually no sialic acid
- Sample D was determined to be partially sialylated, containing approximately 15-35 % sialylated glycans

Generally groups used some form of anion exchange chromatography (DEAE) to determine the percentage of sialylated glycans and, where another technique was applied, the overall number of sialic acid-containing glycans varied relatively little to the rest of the data set, particularly for samples A and B. As can be seen from Figure 17, sample D showed the greatest degree of variation in the number of sialylated structures detected. Nonetheless, as expected on the basis of the glycoprotein source, participants correctly differentiated between highly sialylated glycans (α -1-acid glycoproteins; samples A and B), glycans having moderate levels of sialylation (IgG; sample D) and glycans bearing very little sialic acids (RNase B; sample C).

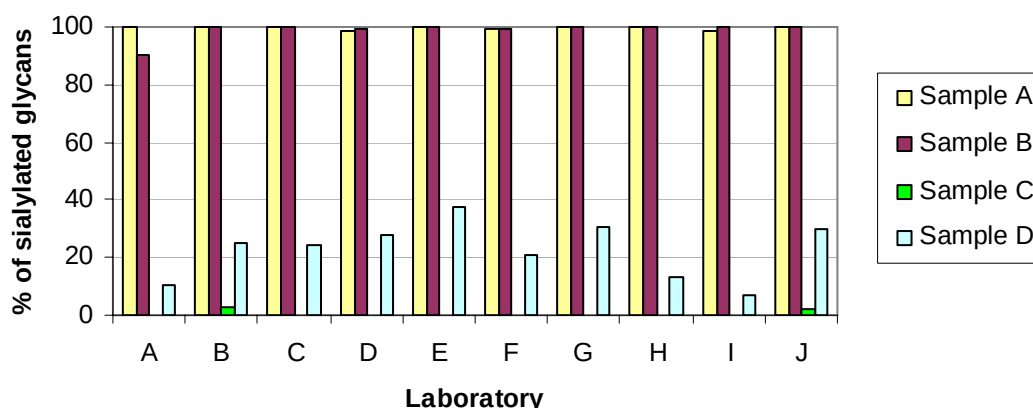


Figure 17: Total percentage of sialylated glycans for samples A, B, C and D

Due to the variation observed for sample D further data analysis was carried out using a scattered plot (Figure 18) based on the overall average percentage of sialylated glycans reported as a benchmark, to help demonstrate the variation amongst participants. The consensus value of the mean percentage, calculated as 22.6 % (solid line), together with a two-fold standard deviation figure of ± 19.6 % (dashed line) was used to derive the plot.

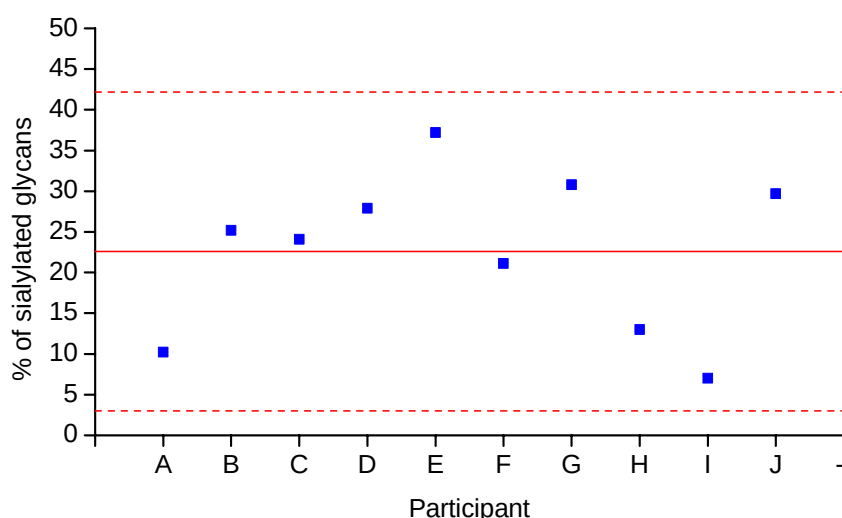


Figure 18: Distribution of the percentage of sialylated glycans reported amongst laboratories for sample D

Despite the variation shown amongst laboratories for the percentage of sialylated glycans, all the data was shown to fall within the two standard deviation lines. Furthermore, the mean total percentage of sialylated glycans estimated in sample D (22.6 ± 9.8 %) was in accord with the literature values on human IgG [133]. Past profiles of oligosaccharides released from polyclonal IgG suggest that the levels of sialic acid observed here may, in fact, be attributed to the 20-30 % of sialylated glycans attached in the Fab region of the IgG molecules [42, 134, 135].

Interestingly, although there were too few participants for good statistics, there was a suggestion that different methods, that is, MS (10.1 ± 3.0 %) compared to chromatography (28.0 ± 5.3 %), gave different mean values. The variation observed

between detection systems may be due to the use of oligosaccharides that were not permethylated in some instances prior to analysis, which would result in differential efficiency in ionisation and fragmentation with MS of the acidic glycans. In a recent study, the analysis of sialylation levels using permethylated glycans by MALDI-MS has been shown to be comparable to chromatographic methods [136].

6.2.1.2 Types of sialylated structures

Samples A and B both contained nearly 100 % of sialylated chains: the consensus was that 99.6 ± 0.6 % and 98.8 ± 3.1 % were sialic acid bearing, respectively. The reported data was evaluated further to obtain additional information about the types of sialylated structures. For instance it is known that samples A (H α 1) and B (B α 1) contain differing amounts of mono-, di-, tri- and tetra-sialylated glycans, and it was envisaged that laboratories would be able to establish a distribution of these. Figure 19 and Figure 20 displays the distribution of the varying proportions of sialylated glycans observed by participants for samples A and B, respectively.

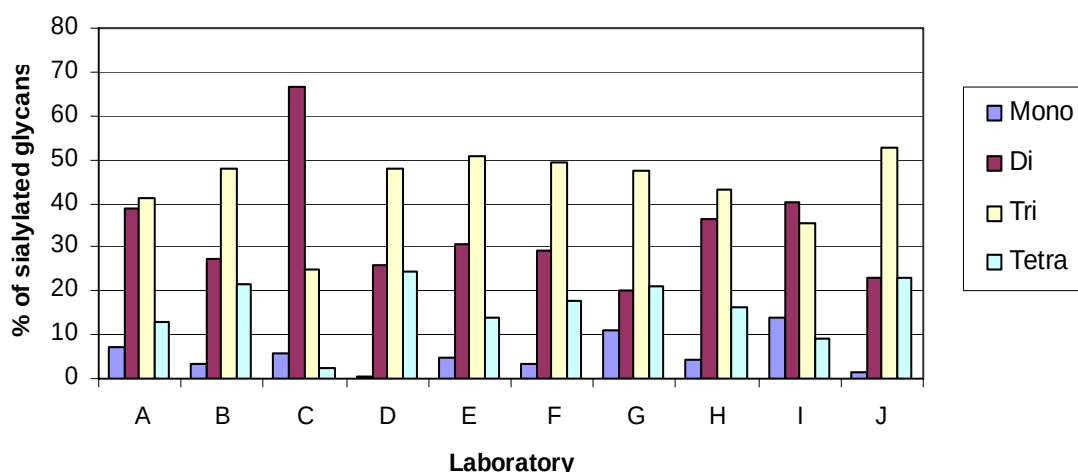


Figure 19: Proportion of sialylated structures found in sample A

As can be seen in the two figures there is a clear pattern between the two different sources of glycans; sample A contained a mixture of all four types of sialylated glycans with di, tri and tetra being the major species. The trend amongst participants for sample A was similar showing in most cases that over 40 % of glycans are tri-sialylated (44.0 ± 8.4 %), although there were a couple of exceptions (Labs C and I showed di- as the major component). The pattern observed for sample B was that di-sialylated structures were the major class (62.1 ± 11.1 %), with no exceptions. The analyses of samples A and B show that, although both are from α -1-acid glycoproteins, the source of these glycoproteins (human or bovine) affects the type of sialylated structures found. It should be noted that estimation of the different types of sialylated structures, for samples A and B, using normal-phase HPLC showed significant differences when compared to analysis by the same laboratory using anion exchange chromatography (data not shown).

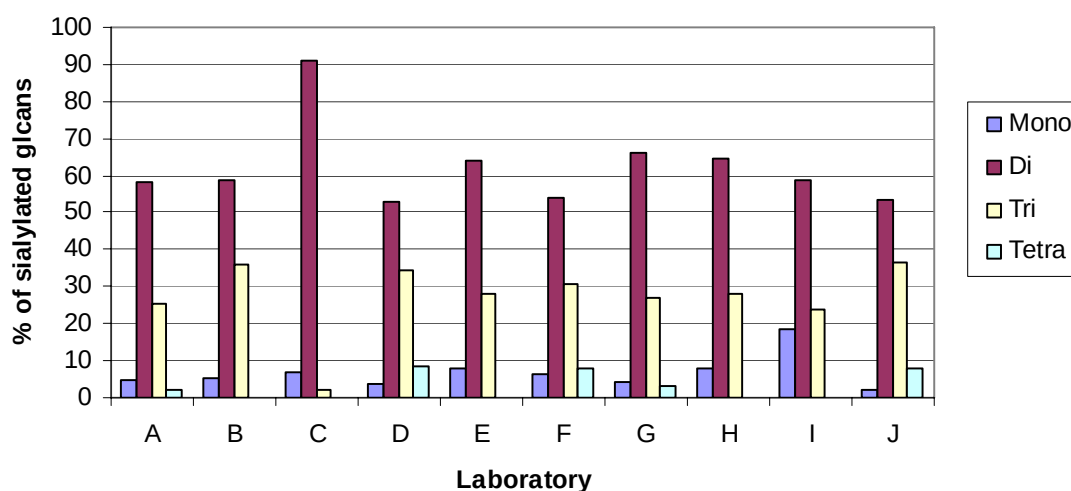


Figure 20: Proportion of sialylated structures found in sample B

Interestingly, only a few participants (four labs) identified that the sialic acids in sample B are a mixture of Neu5Ac and Neu5Gc. Each of the four labs made this observation using mass spectroscopic techniques by detecting a molecular mass of 16 atomic mass units greater for Neu5Gc over that of Neu5Ac. Participants did not state the presence of the non-human sialic acid for amide chromatography-based methods: in theory this is feasible with simple glycan mixtures through the use of reference materials. Detection of Neu5Ac and Neu5Gc can also be carried out using HPLC by labelling the sugars with 1,2-diamino-4,5-(methylenedioxy)benzene (DMB) followed by separation on a C18 column [137]: a method not employed in this study. There is inconclusive evidence within this data set as to whether labelling of the glycans, either through a fluorophore or through some form of methylation, aided the detection of Neu5Gc compared to underivatized glycans. However, it was noted that participants in general generated better spectra with derivatised glycans for the profiling of the two highly sialylated glycans A and B.

Analysis of the sialylated glycan structures found in sample D (IgG) showed that only mono- and di-sialylated bi-antennary oligosaccharides were present, with glycans containing one sialic acid (mono) the prevalent type for each laboratory (Figure 21). Mean calculated values for mono- and di-sialylated glycans were 15.7 ± 6.6 % and 6.9 ± 3.9 %, respectively.

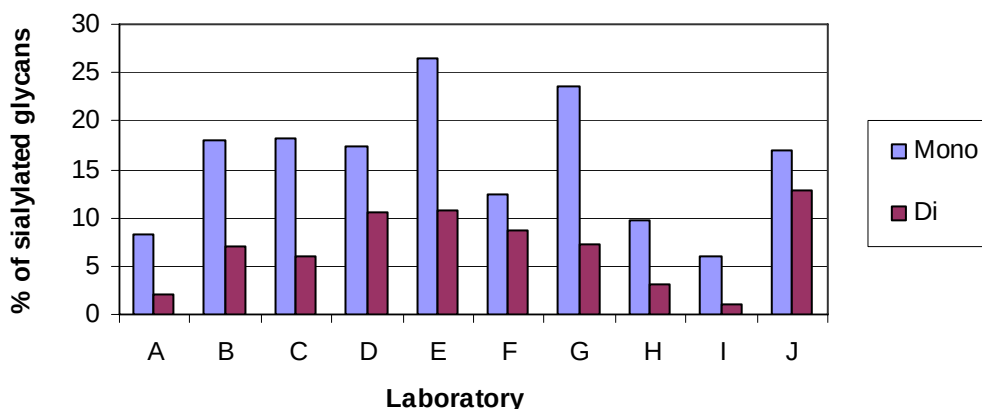


Figure 21: Type of sialylated structures found in sample D

The ratio of mono- and di-sialylated glycans evidently varied between each laboratory and is summarised in Table 6. The average ratio within the data set was 2.9 ± 1.3 (mean $\pm$ standard deviation), with a large CV value of 45.8 %. No pattern was established between chromatographic and mass spectroscopic methodologies with respect to the ratio of sialylated structures.

Table 6: Ratio of mono- and di-sialylated glycans in sample D

Participant	A	B	C	D	E	F	G	H	I	J
Mono/Di ratio	4.0	2.5	3.1	1.6	2.4	2.6	3.2	3.1	6.0	1.3

In order to evaluate the overall charge (Z), a hypothetical parameter characteristic of all charged glycans, the total charge of the samples A, B and D were determined from their sialic acid profiles. Z is defined as the sum of the products of the respective percentages of mono-, di-, tri- and tetra-sialylated glycans, each multiplied by its corresponding charge:

$$(\% \text{ mono} \times 1) + (\% \text{ di} \times 2) + (\% \text{ tri} \times 3) + (\% \text{ tetra} \times 4) = Z$$

Thus, a glycoprotein with mainly bi-antennary sialylated structures will have a Z value of about 200, whilst for a tetra-antennary structure the value Z will be around 400. The Z values for laboratories A to J are depicted in Figure 22. As expected α -1-acid glycoprotein obtained from humans (sample A) gave a higher mean Z number than the bovine material (sample B); 271 ± 24 and 224 ± 18 , respectively. Sample D (IgG) was determined to have a mean Z number of 29 ± 14 . Surprisingly sample D, an industrially relevant material, displayed the greatest variation in the Z number amongst the laboratories with a CV value of 47.3 % compared to values of 8.9 % and 8.1 % respectively, for glycans A and B.

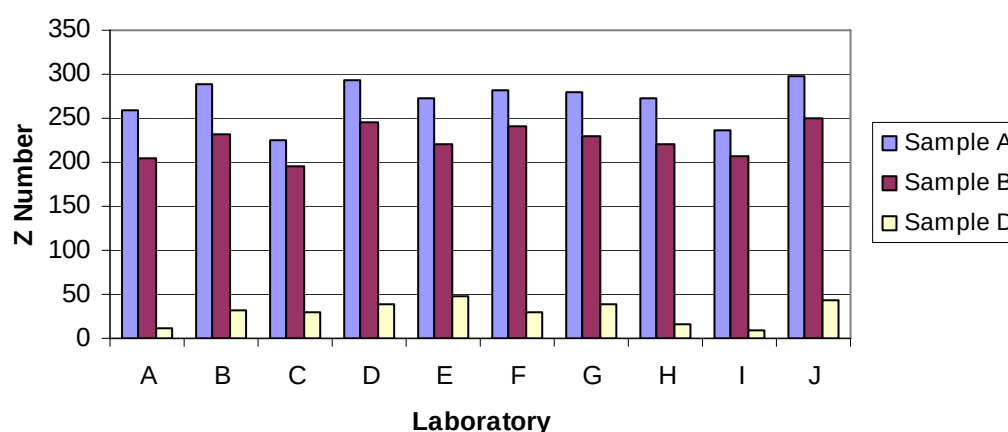


Figure 22: Z number for the sialylated samples A, B and D

In conclusion, the variation in the relative percentages of sialylated structures observed for samples A, B and D demonstrates the importance of using appropriate methodologies for their identification.

6.2.2 Bi-, tri- and tetra-antennary profiles (samples A and B)

The data received from participants to determine the antennary profiles of the samples was rather limited due to the level of detail provided. Therefore the following section only discusses the results where sufficient information could be obtained from the data presented by participants.

Figure 23 represents the antennary profile for the highly sialylated sample A. This material was determined to contain a mixture of bi-, tri- and tetra-antennary glycans with the tri-antennary structure the major species in most cases. The range of material analysed included underivatised, permethylated, fluorophore labelled and desialylated glycans, with HPLC and MALDI-TOF MS the two main techniques employed by participants.

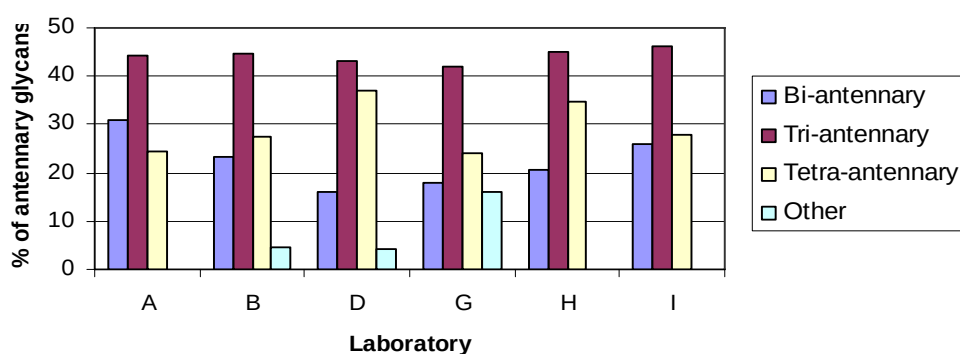


Figure 23: Antennary profile for sample A

Figure 24 outlines the antennary profile for sample B, the other highly sialylated glycan mixture. In this sample bi-antennary sugars were the prevalent class, with tetra-antennary structures not observed. “Other” refers to the structures that were either of the hybrid type or ones that were unidentified.

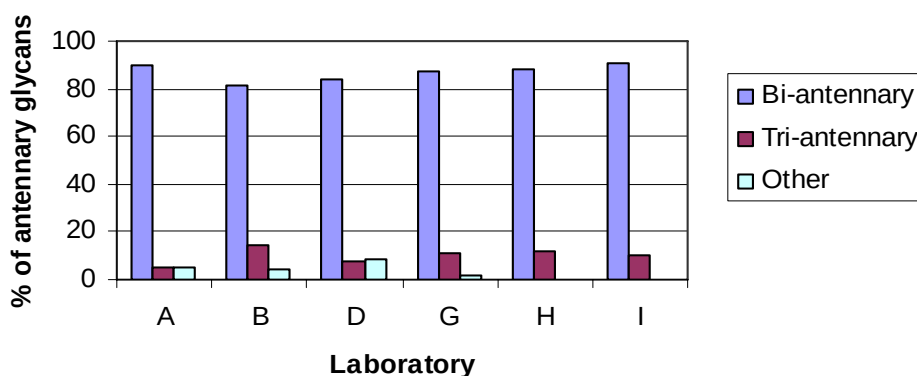


Figure 24: Antennary profile for sample B

Table 7 portrays the standard deviation and the mean percentage of the antennary profiles found by participants for samples A and B. All values for standard deviation were below $\pm 10\%$, the mean and median were in close proximity whilst the CV for sample B showed good agreement within the data set with a value of 4.2 %.

Table 7: Statistical analysis of the antennary profiles for samples A and B

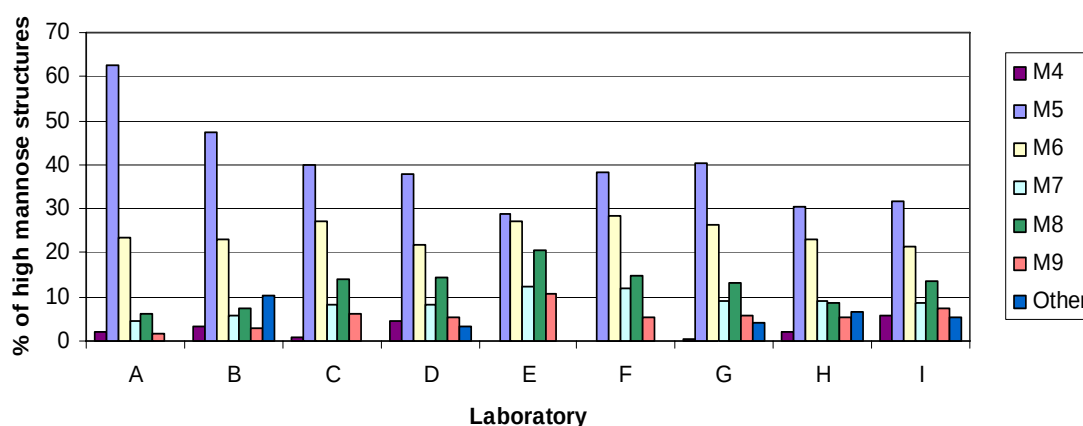
Antennary Structure (n = 6)	Min (%)	Max (%)	Mean (%)	Median (%)	Standard Deviation	CV (%)
Sample A						
Bi-	15.9	31.1	22.5	22.0	± 5.6	25.0
Tri-	41.8	46.0	44.1	44.5	± 1.5	3.3
Tetra-	24.2	36.9	29.2	27.6	± 5.3	18.1
Sample B						
Bi-	81.3	90.7	86.8	87.8	± 3.7	4.2

n = number of laboratories

Thus, in summary, data from glycan analysis illustrates that sample A is highly branched consisting of a mixture of di-, tri- and tetra-antennary glycans while sample B is mainly bi-antennary with a high abundance (86.8 ± 3.7 %) of disialylated oligosaccharides.

6.2.3 Analysis of the neutral *N*-glycans mixture (sample C)

As confirmed by the participating laboratories, the *N*-glycans in sample C were primarily neutral oligosaccharides of the high-mannose type, structurally in agreement with its source RNase B. A comprehensive breakdown (where possible) of the different mannose chains found by laboratories is shown in Figure 25.

**Figure 25: Mannose (neutral glycan) profile of sample C**

Results from each of the nine sets of data, estimated that the major glycan in the sample mixture was composed of five mannose units (M5) and oligosaccharides with six mannose residues (M6) was the second largest group. The minor glycans found amongst participants were those containing four mannose units (M4). A noticeable trend was that, where mass spectroscopic based techniques were used, laboratories observed that sample C contained high mannose *N*-glycans frequently lacking an *N*-acetylglucosamine residue thus, indicating the presence of the *endo*-glycosidase Endo-H in the PNGase F enzyme source. Laboratories B, D, G, H and I also found that in general, apart from the expected high mannose structures there was less than 10.5 % complex/hybrid type *N*-glycans “other” in the sample mixture.

The variability of the high mannose glycans present was analysed further using the mean percentage of each glycan derived from the data, and their respective standard deviations. Table 8 summaries the values obtained for M4, M5, M6, M7, M8 and M9 while Figure 26 shows a plot of the most abundant M5 glycans in relation to the mean (39.7 %) and two-fold standard deviation (± 20.6 %). All values obtained were calculated using the consensus participant data.

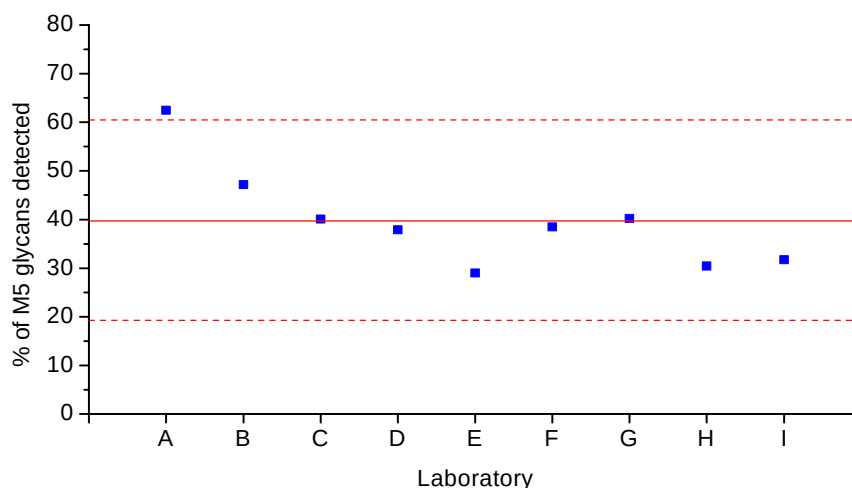


Figure 26: Percentage of M5 glycans found in sample C

The plot illustrates that all data points except for one which lies between two standard deviations (± 20.6 %) (dashed line) of the mean. In fact all but one value is within one standard deviation (± 10.3 %) of the mean.

Table 8: Summary of the high-mannose structures found on sample C

(n = 9)	Min (%)	Max (%)	Mean (%)	Median (%)	Standard Deviation	CV (%)
M4	0	5.6	2.1	1.9	± 2.0	95.6
M5	29.0	62.5	39.7	38.5	± 10.3	25.9
M6	21.3	28.4	24.7	23.4	± 2.6	10.5
M7	4.4	12.5	8.7	8.6	± 2.6	29.7
M8	6.0	20.4	12.5	13.7	± 4.4	35.6
M9	1.8	10.8	5.7	5.5	± 2.6	45.5

n = number of laboratories

The largest variation observed for the high mannose oligosaccharides was that for M5 with a deviation of ± 10.3 % with M4 having the smallest deviation. On the other hand when comparing the coefficient of variation (CV) unsurprisingly M4 has the largest value due to its relative low abundance while M6 has the smallest at 10.5 %. The mean and median percentages were comparable. In general, regardless of the standard deviation values being below ± 10 % (with the exception of one) the CV figures are large, demonstrating a sizeable variability amongst the data set. The variations observed were surprising considering that this particular sample was predicted to be the easiest to identify and to also give the least dissimilarity as it consists of neutral N-glycans. Unfortunately, due to the small sample pool the variation observed could not be attributed to any specific technique.

6.2.4 Galactose, fucose and bisecting GlcNAc profiles (sample D)

Unsurprisingly participants found that sample D, which was prepared from polyclonal human IgG, consisted mainly of complex bi-antennary structures (<89 %) with varying amounts of galactosylation. Laboratories D, H and I also reported the presence of the high mannose and/or hybrid oligosaccharide chains ranging from 1.8-9.0 %. A small percentage of tri-antennary structures (<2.5 %) were also found by the above respondents. The level of galactosylation (G0, G1 and G2) is often employed in industry to monitor the glycosylation pattern of recombinant monoclonal antibodies. This profiling system is also used to examine the state of a disease, such as rheumatoid arthritis, through changes in IgG glycosylation [135]. Thus, the bi-antennary structures were evaluated further, where possible, to investigate their galactosylation profile and the results from nine laboratories are displayed in Figure 27. For laboratories that distinguished between the two different monogalactosylated isomers the values were combined and represented as G1.

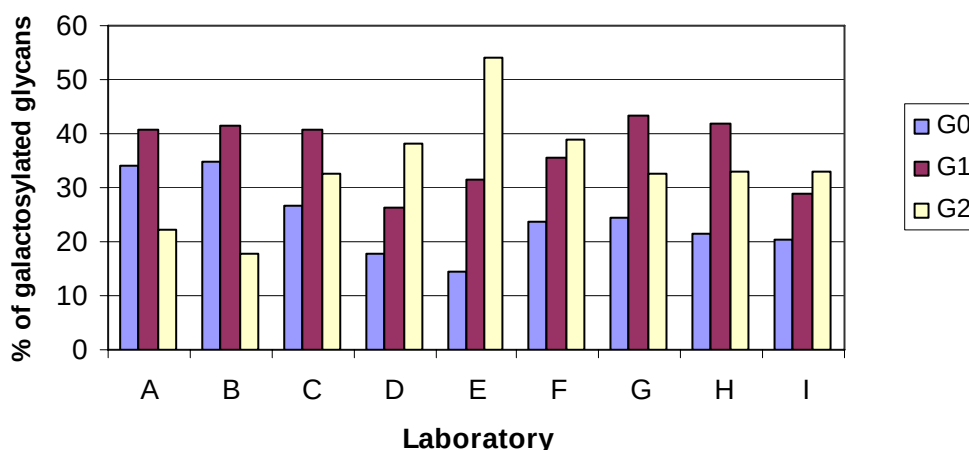


Figure 27: Galactosylation profile of sample D

The ratios of G0, G1 and G2 terminal galactose bi-antennary structures reported varied from participant-to-participant: some had higher levels of G0 compared to G2 and in other examples this trend was reversed. The levels of G0 found also varied from 14 % to 35 %. These inconsistencies may in part be accounted for by the range and level of information obtained by the techniques utilised by laboratories, with the two main sources of feedback arriving from MS and HPLC methodologies. Another source of variation may be due to the determination of G0/1/2 before and after desialylation, but in the case out of the above data set, only one laboratory reported the galactosylation profile after desialylation and no large variation was calculated.

Table 9: Galactosylated structures in sample D

Structure (n=9)	Min (%)	Max (%)	Mean (%)	Median (%)	Standard Deviation	CV (%)
G0	14.5	34.9	24.1	23.8	± 6.8	28.1
G1	26.3	43.3	36.3	36.6	± 6.2	17.0
G2	17.7	54.0	34.2	33.0	± 10.4	30.4

n = number of laboratories

The table above attempts to deconvolute the variation observed for the terminal galactose units present in sample D (Table 9). No clear distinction between the instrument used and the level of G0, G1 and G2 structures observed could be established within the data set.

Additional oligosaccharide structures generally found on IgG include core-linked fucose and bisecting GlcNAc. The application of non-fucosylated therapeutic antibodies is of interest to industry, as this form is known to enhance ADCC activity [45, 138], whilst the presence of bisecting GlcNAc inhibits the addition of fucose [139]. Hence, the additional structures present in sample D were evaluated, where sufficient data was available, and their relative abundance is shown in Figure 28. The presence of bisecting GlcNAc was calculated to be significantly lower in all cases in comparison to the core-linked fucosyl moiety. Mean relative percentage of GlcNAc and fucose found were 16.9 ± 5.8 % and 80.9 ± 12.5 %, respectively.

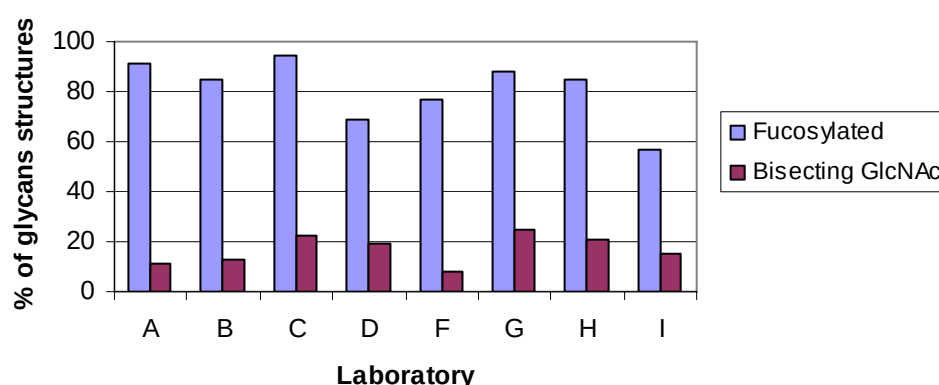


Figure 28: Additional glycan structures found in sample D

The ratios of fucosylated and bisecting GlcNAc species found in the three different galactosylated structures are shown in Table 10. For the fucosylated glycans the mean ratio of galactosylation/fucosylation decreased from 1.07 ± 0.05 to 0.78 ± 0.19 as the level of galactosylation increased. On the other hand, there was an increase in the mean ratio of galactosylation/bisecting GlcNAc as the number of terminal galactosyl residues increased. CV values were all greater than 10 % except for one. In summary, sample D consisted of highly fucosylated bi-antennary structures, with varying levels terminal galactosyl residues, which partially contain bisecting GlcNAc units.

Table 10: Ratios of fucose and bisecting GlcNAc found in sample D

Structure	Min	Max	Mean	Standard Deviation	CV (%)
Fucose (n = 8)					
G0/G0f	1.00	1.14	1.07	± 0.05	4.3
G1/G1f	0.73	1.10	0.92	± 0.11	11.7
G2/G2f	0.46	1.04	0.78	± 0.19	24.6
GlcNAc (n = 7)					
G0/(G0fb+G0b)	3.56	9.69	5.37	± 2.13	39.7
G1/(G1fb+G1b)	4.08	9.09	5.50	± 1.81	33.0
G2/(G2fb+G2b)	3.72	11.06	6.22	± 2.46	39.5

n = number of laboratories

6.2.5 Comparison of glycan mapping techniques

The number of distinct carbohydrate structures identified correlates to the techniques used by the laboratories: the information was derived by adding up the number of glycans identified on the glycan analysis spreadsheet, and is portrayed in Figure 29. For all four samples, more *N*-glycans were identified with mass spectroscopic-based techniques (analyses 1 to 12) compared to HPLC (analyses 13 to 16). These differences could be attributed to better separation and detection of glycans. Notably, it was recognized that, analysis using a combination of chromatographic- and mass spectroscopic-based methods (analyses 8 and 9) allowed detection of the greatest numbers of glycan structures compared to other approaches.

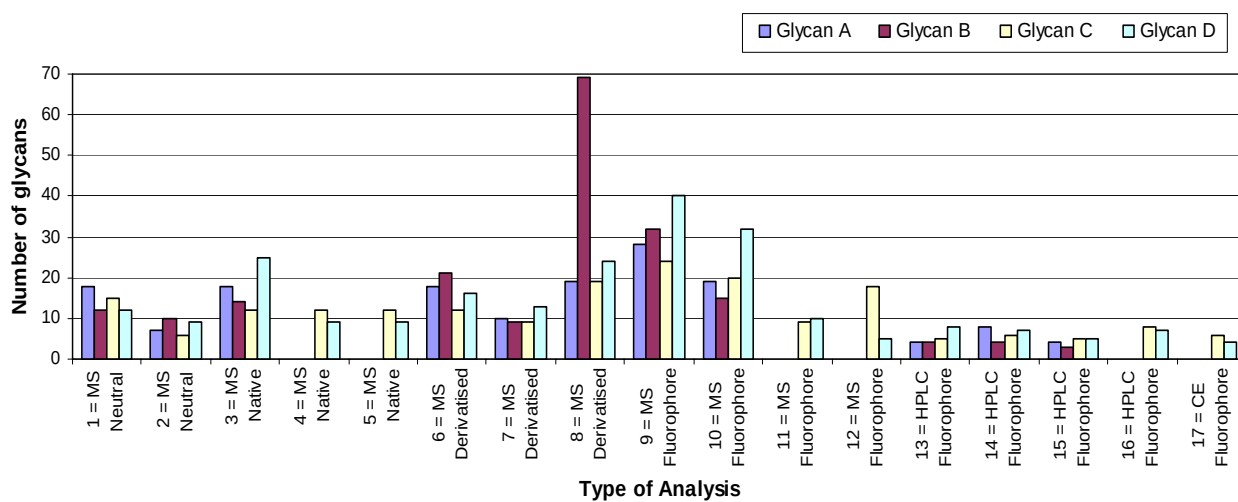


Figure 29: Total number of glycans identified

Of the four samples, A and B appear to have been most problematic to probe, since not all laboratories successfully reported similar numbers of glycans found for these two materials (analyses 4, 5, 11, 12, 16 and 17). This may have been due to the highly sialylated nature of these samples which resulted in difficulties arising during their analyses. For example, it is known that the ionisation of charged glycans is suppressed when using MALDI-TOF MS and different modes (negative or positive) of detection will give different spectra. The lability of sialic acid ketosidic linkages may also have hindered detection. The high number of glycans found in sample B for analysis 8 (MS with derivatised sugars) may be accounted for by the identification of numerous combinations of Neu5Ac and Neu5Gc residues and different linkages of sugars. Overall, MS-based techniques detected 17 ± 6.3 and 23 ± 20.1 glycans for samples A and B respectively, whilst HPLC produced 5 ± 2.3 and 4 ± 0.6 , respectively (Table 11). The data obtained from analyse using a combination of both MS and HPLC was insufficient and therefore results from this are not discussed.

Table 11: Comparison of the number of glycans found with MS alone and HPLC

	A	B	C	D
MS	17 ± 6.3	23 ± 20.1	14 ± 5.3	17 ± 10.8
HPLC	5 ± 2.3	4 ± 0.6	6 ± 1.4	7 ± 1.3

Samples C and D, the neutral and the less sialic acid populated glycans, gave six additional data sets. Despite this more oligosaccharides were observed by MS (14 ± 5.3 and 17 ± 10.8) than by HPLC (6 ± 1.4 and 7 ± 1.3) for C and D, respectively.

In mass spectrometric evaluations a range of oligosaccharides were analysed: underivatised (native and neutral), permethylated, fluorophore labelled and sialic acid methyl ester glycans. The general trend overall was that sample derivatisation enhanced detection of glycans. One group investigated the glycan structures of underivatised material using three different matrices: CHCA (α -cyano-4-hydroxy cinnamic acid), DHB (2,5-dihydroxybenzoic acid) and THAP (2,4,6-trihydroxyacetophenone). DHB showed good resolution, compared to the other matrices, for the neutral (sample C) and partially sialylated (sample D) materials. Spectral resolution was poor for all three matrices in the case of the highly acidic samples (A and B).

Interestingly, one group identified, through *exo*-glycosidase digestions and MS, the 2-3 and 2-6 linkage positions of the sialic acid residues. The same group also distinguished between the different linkages found in the high-mannose glycans (sample C). One group also performed analysis *via* methylation followed by hydrolysis of the glycans using GC-MS. There was no correlation between the level of structural information received about the glycan structures or the number of glycans found, and the quality system employed by the organisation. Unfortunately, further conclusions were limited due to the broad spread of data across various methodologies, as can be observed from Figure 29. Furthermore, due the range of practices employed in the study, no conclusive conclusions could be obtained for the different fluorophore labels utilised.

7.0 EMERGING TECHNIQUES IN GLYCAN ANALYSIS

The development of new tools and techniques, including continued improvements in the accuracy, sensitivity and precision of glycan analysis, is desirable to advance our understanding of and control of glycosylation. The present emphasis is on rapid, reliable high-throughput analysis using MS-based techniques.

An alternative approach currently being explored is the use of lectin-based arrays for the rapid glycoprofiling of therapeutics during process development and manufacturing. The platform is based on the binding of intact glycoproteins to immobilised lectins on a chip, hence omitting the time-consuming step of releasing and purifying the glycans, which is required for most chromatography and MS procedures. The binding of the glycoprotein to the array generates a characteristic fingerprint that is sensitive to changes in glycosylation. Fluorophore-labelled antibodies against the protein moiety or other lectins can be used for detection. The applicability of the array platform has been demonstrated through the analysis of four recombinant mAbs with HPLC [140]. The general consensus is that this emerging method and those based on high-throughput MS will complement, rather than replace, existing technologies such as chromatography and MS methods. The ability of synthetic chemists to produce fully defined glycan structures is also increasing the number of well-characterised reference materials available.

Other areas where improvements are being made are in the availability of glycoinformatic tools and in such a highly complex field, the lack of glycoinformatics tools in glycoanalysis is a problem. Some tools are available [141-144], such as a commercial glycan structure databases (see www.glycosuite.com, www.eurocarbdb.org, www.glycan.genome.jp and [144]www.functionalglycomics.org), as well as computer programs for mass spectrometric analysis of glycans (see www.eurocarbdb.org and www.expasy.org). However, these have limitations and do not address the problem of integrating and processing considerable amount of complex data. A good set of glycoinformatic tools would not only solve the problem of inconsistent nomenclature for glycans, but also enable comparison of data from a wide range of analytical methods.

8.0 CONCLUSIONS

This inter-laboratory study, demonstrates clearly that the analysis of oligosaccharides by participants exploited a range of approaches, with the level of accuracy and information desired being a key factor in the choice of the techniques employed. Trends in the study demonstrate that chromatographic and mass spectroscopic techniques are the methods of choice, with anion exchange chromatography frequently employed to investigate the sialic acid/charge profile of glycans. In general, participants employed at least two techniques to characterise the glycan samples supplied to them. In some instances analysis by chromatography was coupled either off-line or on-line to MS.

Glycans were, by and large, identified *via* the molecular weight of individual glycans using MS. Identification of the glycan mixtures was also achieved with MS/MS experiments: fragmentation patterns of selected oligosaccharides were performed to acquire detailed knowledge about their sugar content, branching and linkages. Where there was ambiguity with MS, *exo*-glycosidase digestion experiments were undertaken to confirm the presence or not of particular glycans. Comparison of retention times or mass peaks with reference compounds and glycan libraries were also explored to identify glycans. However, this latter approach produced the greatest variation in glycan identification. Overall, participants preferred to use more than one method to identify glycans.

Of the glycan samples subjected to analysis, samples C and D proved less difficult to analyse than the highly sialylated samples A and B. The variations observed for the various sialylated, high-mannose and IgG structures clearly emphasise the need to employ the appropriate technology/method to elucidate glycoprofiles. Of particular note is the observation that, although MS enabled a greater number of glycans to be identified compared to HPLC, the percentage of sialylated glycans detected by this technique was lower than with anion exchange chromatography. The need for the use of appropriate techniques is further exemplified by the broad range of values reported by participants for the - all important - G0/G1/G2 profile in sample D. Nonetheless, given the variety of methods used in the study the overall data was generally in good agreement with expected values.

Unfortunately the broad range of methods considered in the present study indicates that a larger data set is required in order to compare further data between particular methods. For example, these might include a range of fluorophore labels, glycans

analysis following desialylation and/or analysis of derivatised and underivatised glycans. A larger data set is currently being generated through the international USP/NIBSC glycan study.

In conclusion, characterising the products of glycosylation is a highly demanding and complex area. Rapid, reliable and quantitative analytical methods are preferred in deciphering glycosylation patterns found on glycoproteins. The absence of method standardisation in glycan analysis stems in part, from the many techniques available for the measurement of the state of glycosylation. In addition, although for each methodology there exist numerous protocols, only limited guidelines are available for a given organisation to choose between these. For example, data generated by HPLC analysis can differ with the buffer systems and even the sample run time. Techniques may also vary in sensitivity for detecting a particular glycan or may even cause degradation of the material. Thus, it is envisaged, that future guidelines in the pharmacopoeias will seek to take account for and to overcome the issues of standardisation for the analysis of cleaved glycans. The provision of better guidance both on the choice of technique(s) and also on the level of characterisation required at the various stages of production will thus be of primary importance.

9.0 ACKNOWLEDGEMENTS

NPL and NIBSC would like to thank the following organisations for their participation and time in the inter-laboratory study:

- Dublin-Oxford Glycobiology Lab
- GSK
- Intertek
- LGC
- Lonza Biologics Plc
- Ludger Ltd
- M-Scan Ltd
- UCB-Celltech
- University of Birmingham

The authors would also like to thank Anna Hills (NPL) and Tina Morris (USP) for advice and useful discussions.

This project is supported by the UK Government's Department of Trade and Industry, as part of the Measurements for Biotechnology Programme (2004-2007).

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11.0 APPENDIX

11.1 Glycan analysis questionnaire

		Date:			
MfB Glycan Analysis Comparison Study Follow-up Questionnaire					
Please complete this form electronically and return to NPL via e-mail to smita.thobhani@npl.co.uk. Please produce one copy for each different technique used in the study.					
LABORATORY INFORMATION					
Organisation:					
Lab Reference: (for organisers only)					
Contact Person:					
How often does your laboratory do glycan analysis?					
Do you have a group dedicated to glycan analysis?					
GLYCAN SEPARATION					
1. What do you separate/analyse?	Underivatised glycans				
	Fluorophore-labelled glycans				
	Other (please specify)				
2. How do you separate/analyse the glycans?	MS (please specify e.g. MALDI, ESI)				
	HPAEC				
	Capillary electrophoresis (CE)				
	HPLC (please specify e.g. RP, NP)				
	Ion exchange (e.g. AEC)				
	HILIC				
	FACE				
	Gel permeation (SEC)				
	Other (please specify)				
3. If you use MS, do you use a matrix?	No				
	Yes (please specify)				
4. If you use fluorescent labelling, which fluorophore do you use?	2-Aminobenzoic acid (2-AA)				
	2-Aminobenzamide (2-AB)				
	4-Aminobenzoic acid (4-AA)				
	2-Aminopyridine (2-AP)				

	Disodium 8-amino-1,3,6-naphthalene trisulfonate (ANTS)	
	2-Amino-9(10H)-acridinone (AMAC)	
	Trisodium 8-aminopyrene-1,3,6 trisulfonate (APTS)	
	Other (please specify)	
5. For fluorescent labelling, do you use a commercial kit?	No	
	Yes (please specify)	
6. If you use HPLC, how do you detect peaks from the column?	PAD	
	PED	
	UV/Vis absorbance	
	Fluorescence	
	LIF	
	Other (please specify)	
GLYCAN IDENTIFICATION		
7. How do you qualify instrument performance?	Reference glycan mixture	
	Dextran ladder	
	Other (please specify)	
8. How do you identify peaks?	Purified commercial reference compounds	
	In-house purified reference compounds	
	Mass spectroscopy	
	Glycosidase digestion	
	Other (please specify)	
9. How do you quantify peaks?	Integration of the trace	
	Other (please specify)	
10. Do you correct quantified peaks for differences in response factor?	No	
	Yes (please specify basis for this)	
11. What values do you report?	% of molar ratio of glycan components	
	Other (please specify)	
QUALITY ASPECTS		
12. Do you routinely qualify your methods?	No	
	Yes, please specify basis for this	
13. Do you routinely validate your methods?	No	
	Yes, please specify e.g. ICH Q2A, USP 1225, EP	
14a. Do you perform	No	

IQ/OQ/PQ on your instrumentation?	Yes, please specify how often	
14b. If yes for question 14a, who performs the IQ/OQ/PQ on your instrumentation?	Staff personnel	
	Manufacturer's personnel	
	Qualified operator	
15. Do you qualify your reagents?	No	
	Yes, please explain	
16. Does your laboratory operate under one of the following quality systems?	No	
	Yes, please specify e.g. cGMP, cGLP, ISO 17025	
Any other information/comments:		
Thank you for taking the time to complete this questionnaire		

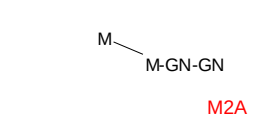
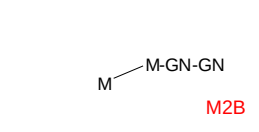
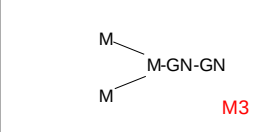
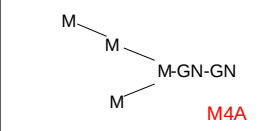
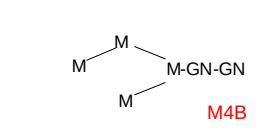
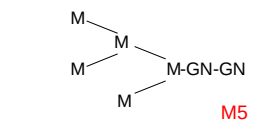
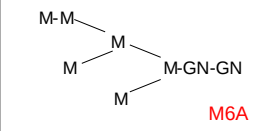
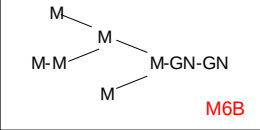
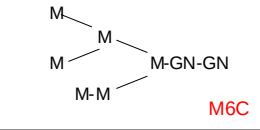
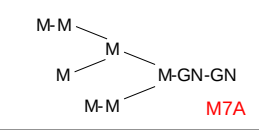
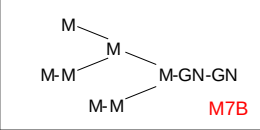
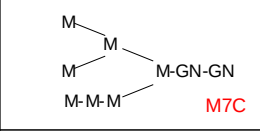
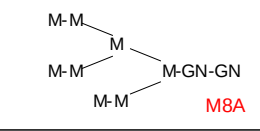
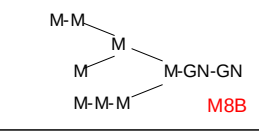
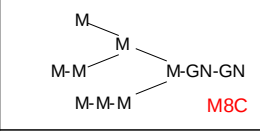
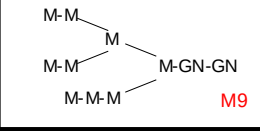
11.2 Standardised reporting data sheet

MfB Glycan Analysis Data Spreadsheet							
Sample A				Sample B			
High mannose chains	% total glycans			High mannose chains	% total glycans		
M1				M1			
M2A			or M2A + M2B	M2A			or M2A + M2B
M2B				M2B			
M3				M3			
M4A			or M4A + M4B	M4A			or M4A + M4B
M4B				M4B			
M5				M5			
M6A			or M6A + M6B + M6C	M6A			or M6A + M6B + M6C
M6B				M6B			
M6C				M6C			
M7A			or M7A + M7B + M7C	M7A			or M7A + M7B + M7C
M7B				M7B			
M7C				M7C			
M8A			or M8A + M8B + M8C	M8A			or M8A + M8B + M8C
M8B				M8B			
M8C				M8C			
M9				M9			
Hybrid chains				Hybrid chains			
(A1)M3(a)			(A1)M3	(A1)M3(a)			(A1)M3
(A1)M3(b)				(A1)M3(b)			
(A1)M3(c)				(A1)M3(c)			
(A2)M3				(A2)M3			
(GN)M4(a)			(GN)M4	(GN)M4(a)			(GN)M4
(GN)M4(b)				(GN)M4(b)			
(LN)M4(a)			(LN)M4	(LN)M4(a)			(LN)M4
(LN)M4(b)				(LN)M4(b)			
(A1)M4(a)				(A1)M4(a)			
(A1)M4(b)				(A1)M4(b)			
(A1)M4(c)			(A1)M4	(A1)M4(c)			(A1)M4
(A1)M4(d)				(A1)M4(d)			
(A1)M4(e)				(A1)M4(e)			
(A1)M4(f)				(A1)M4(f)			
(A2)M4(a)			(A2)M4	(A2)M4(a)			(A2)M4
(A2)M4(b)				(A2)M4(b)			
(GN)M5				(GN)M5			
(LN)M5				(LN)M5			
(A1)M5(a)			(A1)M5	(A1)M5(a)			(A1)M5
(A1)M5(b)				(A1)M5(b)			
(A1)M5(c)				(A1)M5(c)			
(A2)M5				(A2)M5			
(A1)M6(a)			(A1)M6	(A1)M6(a)			(A1)M6
(A1)M6(b)				(A1)M6(b)			
(A2)M6(a)			(A2)M6	(A2)M6(a)			(A2)M6
(A2)M6(b)				(A2)M6(b)			
(A1)M7(a)			A1)M7	(A1)M7(a)			A1)M7
(A1)M7(b)				(A1)M7(b)			
(A2)M7				(A2)M7			
Complex biantennary chains				Complex biantennary chains			
(GN)M2(a)			(GN)M2	(GN)M2(a)			(GN)M2
(GN)M2(b)				(GN)M2(b)			
(GN)M3(a)			(GN)M3	(GN)M3(a)			(GN)M3
(GN)M3(b)				(GN)M3(b)			
NGA2 or G0				NGA2 or G0			
NA1(a) or G1(a)			NA1 or G1	NA1(a) or G1(a)			NA1 or G1
NA1(b) or G1(b)				NA1(b) or G1(b)			
(LN)M3(a)			(LN)M3	(LN)M3(a)			(LN)M3
(LN)M3(b)				(LN)M3(b)			
NA2 or G2				NA2 or G2			
NGA2F or G0f				NGA2F or G0f			
NA1F(a) or G1f(a)			NA1F or G1f	NA1F(a) or G1f(a)			NA1F or G1f
NA1F(b) or G1f(b)				NA1F(b) or G1f(b)			
NA2F or G2f				NA2F or G2f			
NGA2FB or G0fb				NGA2FB or G0fb			
NA1FB(a) or G1fb(a)			NA1FB or G1fb	NA1FB(a) or G1fb(a)			NA1FB or G1fb
NA1FB(b) or G1fb(b)				NA1FB(b) or G1fb(b)			
NA2FB or G2fb				NA2FB or G2fb			
A1(a) or G1S1(a)			A1(a)/A1(b) or G1S1	A1(a) or G1S1(a)			A1(a)/A1(b) or G1S1
A1(b) or G1S1(b)				A1(b) or G1S1(b)			
A1(c) or G2S1(a)			A1(c)/A1(d) or G2S1	A1(c) or G2S1(a)			A1(c)/A1(d) or G2S1
A1(d) or G2S1(b)				A1(d) or G2S1(b)			
A2 or G2S2				A2 or G2S2			
A1F(a) or G1FS1(a)			A1F(a)/A1F(b) or G2FS1	A1F(a) or G1FS1(a)			A1F(a)/A1F(b) or G2FS1
A1F(b) or G1FS1(b)				A1F(b) or G1FS1(b)			
A1F(c) or G2FS1(a)			A1F(c)/A1F(d) or G2FS1	A1F(c) or G2FS1(a)			A1F(c)/A1F(d) or G2FS1
A1F(d) or G2FS1(b)				A1F(d) or G2FS1(b)			
A2F or G2FS2				A2F or G2FS2			
A1FB(a) or G1fbS1(a)			A1FB(a)/A1FB(b) or G2fbS1	A1FB(a) or G1fbS1(a)			A1FB(a)/A1FB(b) or G2fbS1
A1FB(b) or G1fbS1(b)				A1FB(b) or G1fbS1(b)			
A1FB(c) or G2fbS1(a)			A1FB(c)/A1FB(d) or G2fbS1	A1FB(c) or G2fbS1(a)			A1FB(c)/A1FB(d) or G2fbS1
A1FB(d) or G2fbS1(b)				A1FB(d) or G2fbS1(b)			
A2FB or G2fbS2				A2FB or G2fbS2			

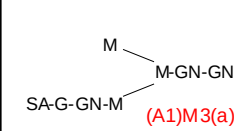
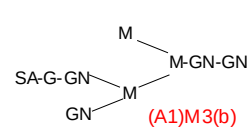
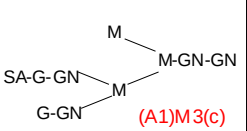
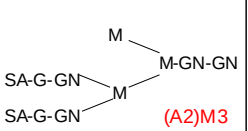
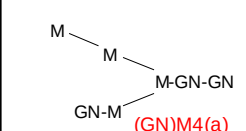
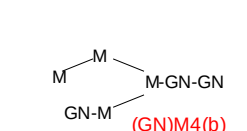
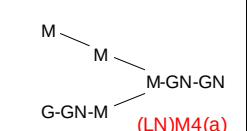
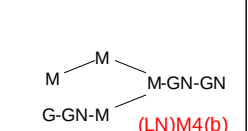
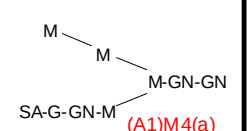
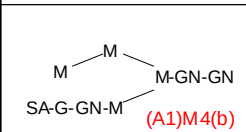
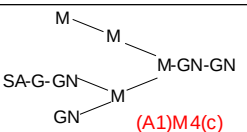
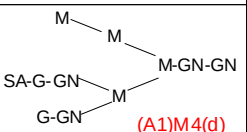
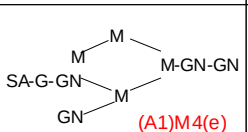
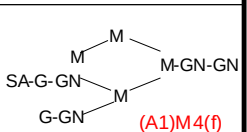
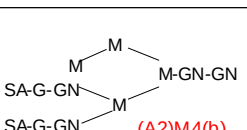
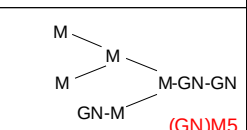
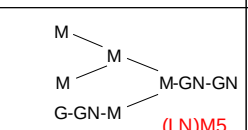
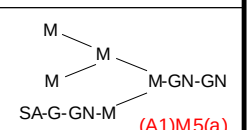
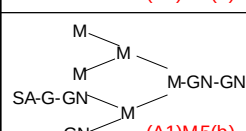
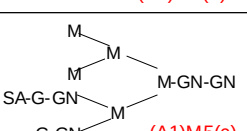
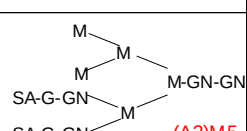
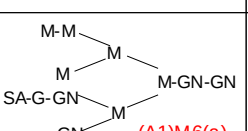
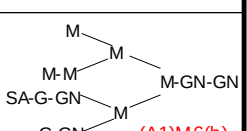
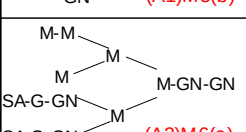
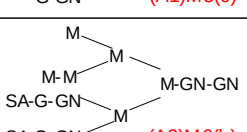
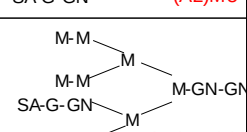
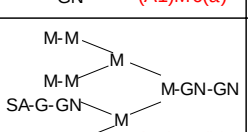
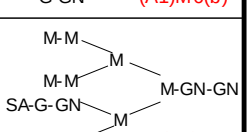
Complex triantennary chains				Complex triantennary chains			
NGA3				NGA3			
NA3(a)				NA3(a)			
NA3(b)			NA3 - 1 Gal	NA3(b)			NA3 - 1 Gal
NA3(c)				NA3(c)			
NA3(d)				NA3(d)			
NA3(e)			NA3 - 2 Gal	NA3(e)			NA3 - 2 Gal
NA3(f)				NA3(f)			
NA3(g)			NA3 - 3 Gal	NA3(g)			NA3 - 3 Gal
NA3S1(a)				NA3S1(a)			
NA3S1(b)			NA3 - 3 Gal - 1 Neu5Ac	NA3S1(b)			NA3 - 3 Gal - 1 Neu5Ac
NA3S1(c)				NA3S1(c)			
NA3S2(a)				NA3S2(a)			
NA3S2(b)			NA3 - 3 Gal - 2 Neu5Ac	NA3S2(b)			NA3 - 3 Gal - 2 Neu5Ac
NA3S2(c)				NA3S2(c)			
A3 or G3S3			NA3 - 3 Gal - 3 Neu5Ac	A3 or G3S3			A3 or G3S3
Complex tetra-antennary chains				Complex tetra-antennary chains			
NGA4				NGA4			
NA4(a)				NA4(a)			
NA4(b)			NA4 - 1 Gal	NA4(b)			NA4 - 1 Gal
NA4(c)				NA4(c)			
NA4(d)				NA4(d)			
NA4(e)				NA4(e)			
NA4(f)			NA4 - 2 Gal	NA4(f)			NA4 - 2 Gal
NA4(g)				NA4(g)			
NA4(h)				NA4(h)			
NA4(i)				NA4(i)			
NA4(j)			NA4 - 3 Gal	NA4(j)			NA4 - 3 Gal
NA4(k)				NA4(k)			
NA4(l)				NA4(l)			
NA4(m)			NA4 - 4 Gal	NA4(m)			NA4 - 4 Gal
NA4S1(a)				NA4S1(a)			
NA4S1(b)			NA4 - 4 Gal - 1 Neu5Ac	NA4S1(b)			NA4 - 4 Gal - 1 Neu5Ac
NA4S1(c)				NA4S1(c)			
NA4S1(d)				NA4S1(d)			
NA4S2(a)				NA4S2(a)			
NA4S2(b)			NA4 - 4 Gal - 2 Neu5Ac	NA4S2(b)			NA4 - 4 Gal - 2 Neu5Ac
NA4S2(c)				NA4S2(c)			
NA4S2(d)				NA4S2(d)			
NA4S3(a)				NA4S3(a)			
NA4S3(b)			NA4 - 4 Gal - 3 Neu5Ac	NA4S3(b)			NA4 - 4 Gal - 3 Neu5Ac
NA4S3(c)				NA4S3(c)			
NA4S3(d)				NA4S3(d)			
A4 or G4S4			NA4 - 4 Gal - 4 Neu5Ac	A4 or G4S4			NA4 - 4 Gal - 4 Neu5Ac
Any additional glycans identified			Please specify	Any additional glycans identified			Please specify
Any identified glycan mixtures			Please specify	Any identified glycan mixtures			Please specify
Any glycans not identified			Please specify	Any glycans not identified			Please specify
TOTAL				TOTAL			
Optional Analysis:	Sample A			Optional Analysis:	Sample B		
% of Sialic acid content				% of Sialic acid content	Not required		
Method used for Sialic acid content				Method used for Sialic acid content	-		

11.3 Glycan structures

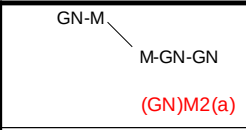
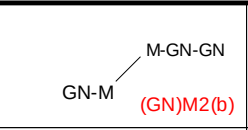
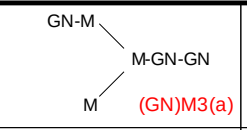
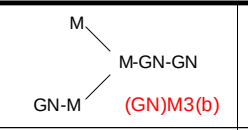
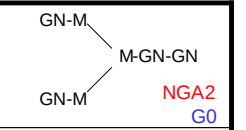
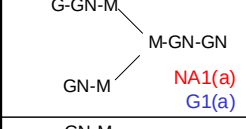
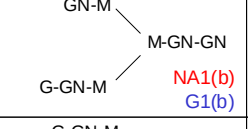
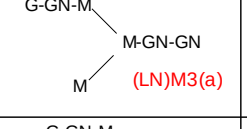
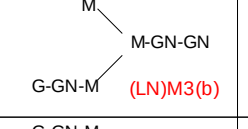
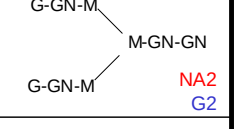
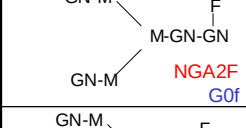
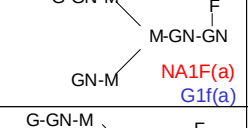
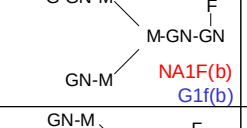
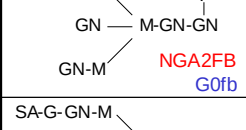
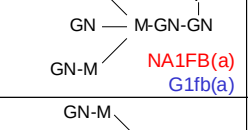
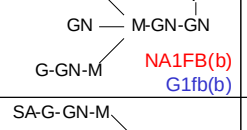
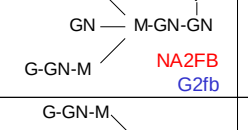
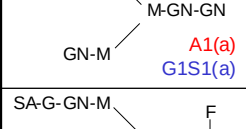
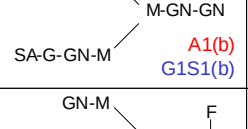
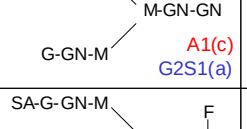
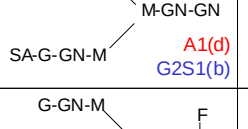
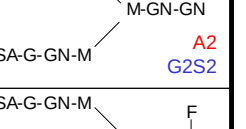
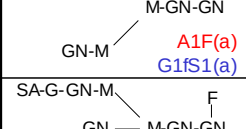
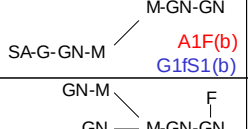
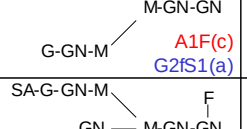
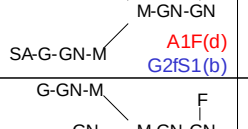
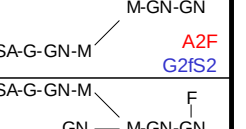
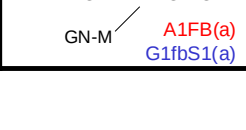
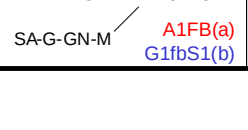
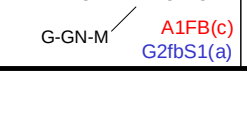
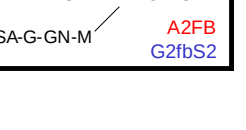
11.3.1 High Mannose Glycan Structures

 M1	 M2A	 M2B	 M3
 M4A	 M4B	 M5	 M6A
 M6B	 M6C	 M7A	 M7B
 M7C	 M8A	 M8B	 M8C
 M9			

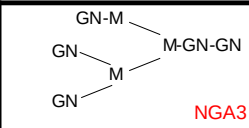
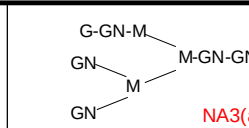
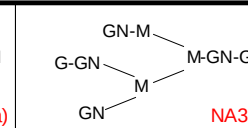
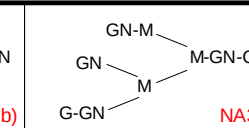
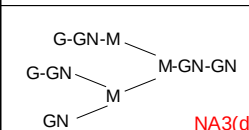
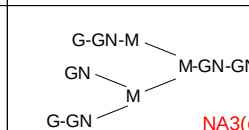
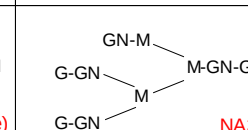
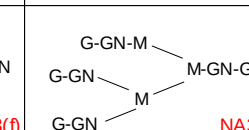
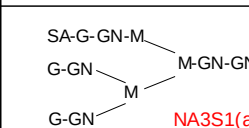
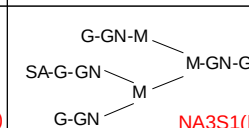
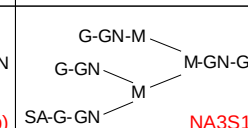
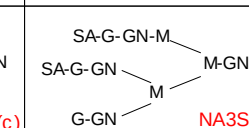
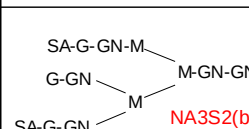
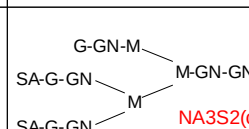
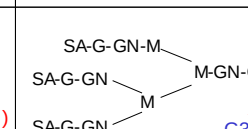
11.3.2 Hybrid Glycan Structures

 (A1)M3(a)	 (A1)M3(b)	 (A1)M3(c)	 (A2)M3	
 (GN)M4(a)	 (GN)M4(b)	 (LN)M4(a)	 (LN)M4(b)	 (A1)M4(a)
 (A1)M4(b)	 (A1)M4(c)	 (A1)M4(d)	 (A1)M4(e)	 (A1)M4(f)
 (A2)M4(a)	 (A2)M4(b)	 (GN)M5	 (LN)M5	 (A1)M5(a)
 (A1)M5(b)	 (A1)M5(c)	 (A2)M5	 (A1)M6(a)	 (A1)M6(b)
 (A2)M6(a)	 (A2)M6(b)	 (A1)M7(a)	 (A1)M7(b)	 (A2)M7

11.3.3 Complex Bi-antennary structures

 (GN)M2(a)	 (GN)M2(b)	 (GN)M3(a)	 (GN)M3(b)	 NGA2 G0
 NA1(a) G1(a)	 NA1(b) G1(b)	 (LN)M3(a)	 (LN)M3(b)	 NA2 G2
 NGA2F G0f	 NA1F(a) G1f(a)	 NA1F(b) G1f(b)	 NA2F G2f	
 NGA2FB G0fb	 NA1FB(a) G1fb(a)	 NA1FB(b) G1fb(b)	 NA2FB G2fb	
 A1(a) G1S1(a)	 A1(b) G1S1(b)	 A1(c) G2S1(a)	 A1(d) G2S1(b)	 A2 G2S2
 A1F(a) G1fS1(a)	 A1F(b) G1fS1(b)	 A1F(c) G2fS1(a)	 A1F(d) G2fS1(b)	 A2F G2fS2
 A1FB(a) G1fbS1(a)	 A1FB(a) G1fbS1(b)	 A1FB(c) G2fbS1(a)	 A1FB(d) G2fbS1(b)	 A2FB G2fbS2

11.3.4 Complex Tri-antennary structures

 NGA3	 NA3(a)	 NA3(b)	 NA3(c)
 NA3(d)	 NA3(e)	 NA3(f)	 NA3(g)
 NA3S1(a)	 NA3S1(b)	 NA3S1(c)	 NA3S2(a)
 NA3S2(b)	 NA3S2(c)	 A3 G3S3	

11.3.5 Complex Tetra-antennary structures
