Development of Tools for Glycan Analysis and Quantitative Sialic Acid Glycomics

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Declaration

The work examined in this thesis is entirely my own. Contributions made by my colleagues are acknowledged in the acknowledgements of assistance. This work has not been, and is not, currently being submitted for candidature for any other degree.
Acknowledgements

Initially, I would like to extend my deepest gratitude to Dr Sarah Allman for her continuous guidance and assistance. I would like to also thank Dr Martin Bootman, for his help and support, and Dr Holger Kramer for his skill and guidance, and for the analysis by nano-LC-MS.

I would also like to express my gratitude to Brett Keith, George Bryant and Matt Kershaw at The Open University and Dr Nicholas Michael at The University of Reading for their assistance in the lab and in performing analysis.

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Abstract

Glycans are molecules consisting of glycosidically linked monosaccharides, ubiquitous in biological systems forming the glyco portion of many glycoconjugates including glycoproteins and glycolipids. The variety in the glycan structure dictate the wide variety of roles these glycans play. These include protective, stabilising and organisation functions. In addition glycans present on biotherapeutics affect the stability and efficacy of the drugs due to the sialylation of the glycan and therefore biotherapeutic glycans must be characterised as their presence is regulated by drug agencies.

The inherent structures of glycans hinders their detection by traditional methods and therefore a derivative of the glycan are often analysed to overcome the poor detection. One of these derivatization methods is where a chemical label is added to the reducing end of the glycans by reductive amination to facilitate detection, however, these modifications prevents further uses of glycans which due to the difficulty of glycan synthesis is a drawback of these workflows. Glycan analysis is commonly performed in hydrophilic interaction mode HPLC, however this method of HPLC suffers from many limitations due to the mechanism of retention relying on an immobilised layer of water. Hydrophilic interaction HPLC suffers from poor peak shape, difficult method development and long equilibration times.

The analysis of glycans is also complicated by the class of monosaccharide, sialic acid which is common on the non-reducing end of the glycans. Sialic acid play many roles in biotherapeutic clearance. For these reasons sialic acid analysis is also performed as the derivative due to the inherent negative charge of sialic acid. Derivatisation is performed with 1,2-diamino-4,5-methylenedioxybenzene (DMB), however this label is associated with poor stability and samples require analysing as soon as possible after labelling.
This thesis presents potential solutions to these observations. The synthesis of two multifunctional glycan labels containing additional groups which show similar HILIC mode HPLC analysis to currently used labels for the analysis of glycans meaning these labels can be employed in glycan analysis workflows to provide further uses. The further uses investigated in this research examined the ability for the carbohydrates labelled with the alkyne multifunctional labels to react with an azide conjugation partners to give a more sensitive molecule to improve detection and characterisation.

This research also investigated the ability for the analysis of glycans via reverse phase HPLC methods to overcome the common limitations of hydrophilic interaction mode HPLC. The reverse phase HPLC of labelled carbohydrates resulted in the production and optimisation of eight reverse phase HPLC methods capable of analysing derivatised glycans from a variety of sources which may be used as alternatives to hydrophilic interaction HPLC for the analysis of glycans.

Finally, this thesis trialled ortho-phenylenediamine derivatives in the quinoxaline derivatisation of sialic acids in an effort to find a possible alternative to DMB in the analysis of sialic acid. These labels were employed in the analysis of Neu5Ac and were observed to provide sufficient detection and resolution for the labelled sialic acid in reverse phase HPLC. These alternatives however were found to be inferior to DMB which displayed similar stability and superior detection sensitivity.
Abbreviation

2-AA  2-anthranilic acid
2-AB  2-aminobenzamide
2-PA  2-aminopyridine
3-AQ  3-aminoquinoline
6-AQ  6-aminoquinoline
AA-Ac 3-(acetylamine)-6-aminoacridine
ABBE  Butyl-4-amino-benzoate
ABEE  Ethyl-4-amino-benzoate
ABME  Methyl-4-aminobenzoate
AMAC  2-aminoacridone
ANSA  5-Amino-2-naphthalene-sulfonic acid
ANTS  8-aminoanaphthalene-1,3,6-trisulfonic acid
APS   Ammonium persulfate
APTS  8-aminopyrene-1,3,6-trisulfonic acid
ATR-IR Attenuated total reflectance infrared spectroscopy
BACH  Biotinamidohexanoic acid hydrazide
BBS   Borate buffered saline
BCN   Bicyclonon-4-yne
BHK   Baby hamster kidney
BOC   tert-butyloxycarbonyl
CCS   Collisional cross section
CE    Capillary electrophoresis
CEM   Chain ejection model
CHO   Chinese hamster ovary
CMAH  CMP-N-acetylneuraminic acid hydroxylase
CRM   Charge residue model
CuAAC Copper catalysed azide-alkyne cycloaddition
Cul   Copper iodide
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<tr>
<td>CV</td>
<td>Column volume</td>
</tr>
<tr>
<td>Da</td>
<td>Dalton</td>
</tr>
<tr>
<td>DCC</td>
<td>N,N'-dicyclocarbodiimide</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DEB</td>
<td>2,3-dihydro-1,4-benzodioxine-6,7-diamine</td>
</tr>
<tr>
<td>DFCO</td>
<td>3,3-difluorocyclooctyne</td>
</tr>
<tr>
<td>DI-MS</td>
<td>Direct injection mass spectrometry</td>
</tr>
<tr>
<td>DMB</td>
<td>1,2-diamino-4,5-methylene-dioxybenzene</td>
</tr>
<tr>
<td>DMP</td>
<td>Dess-Martin periodinane</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DMT-MM</td>
<td>4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium</td>
</tr>
<tr>
<td>ECI</td>
<td>Extracted ion chromatogram</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>EPO</td>
<td>Erythropoietin</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionisation</td>
</tr>
<tr>
<td>EU</td>
<td>Emission units</td>
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<td>FA</td>
<td>Formic acid</td>
</tr>
<tr>
<td>FLD</td>
<td>Fluorescence detection</td>
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<tr>
<td>FSA</td>
<td>Free sialic acid</td>
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<td>GAGs</td>
<td>Glycosaminoglycans</td>
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<tr>
<td>GaINAc</td>
<td>N-acetylgalactosamine</td>
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<tr>
<td>GHP</td>
<td>Glucose homopolymers</td>
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<tr>
<td>GlcNAc</td>
<td>N-acetylglucosamine</td>
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<tr>
<td>GPI</td>
<td>Glycosylphosphatidylinositol</td>
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<tr>
<td>GU</td>
<td>Glucose units</td>
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<td>HILIC</td>
<td>Hydrophilic interaction liquid chromatography</td>
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<tr>
<td>HOMO</td>
<td>Highest occupied molecular orbital</td>
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<tr>
<td>HMBC</td>
<td>Heteronuclear multiple bond correlation</td>
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<td>HPAEC</td>
<td>High pH anion exchange chromatography</td>
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<td>Acronym</td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<td>hRuEPO</td>
<td>Human recombinant erythropoietin</td>
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<td>HRMS</td>
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<td>HSQC</td>
<td>Heteronuclear single quantum coherence</td>
</tr>
<tr>
<td>ID</td>
<td>Internal diameter</td>
</tr>
<tr>
<td>IEEDA</td>
<td>Inverse electron-demand Diels-Alder</td>
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<tr>
<td>IEM</td>
<td>Ion evaporation model</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>KDN</td>
<td>2-keto-3-deoynonic acid</td>
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<td>LIF</td>
<td>Light induced fluorescence</td>
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<td>LLE</td>
<td>Liquid-liquid extraction</td>
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<td>LLOD</td>
<td>Lower limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantitation</td>
</tr>
<tr>
<td>LTQ</td>
<td>Linear Trap Quadrupole</td>
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<tr>
<td>LUMO</td>
<td>Lowest unoccupied molecular orbital</td>
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<tr>
<td>mAbs</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MALDI</td>
<td>Matrix assisted laser desorption ionisation</td>
</tr>
<tr>
<td>MeCN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>MWCO</td>
<td>Molecular weight cut off</td>
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<tr>
<td>NEt₃</td>
<td>Triethylamine</td>
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<tr>
<td>Neu5Ac</td>
<td>N-acetylneuraminic acid</td>
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<td>Neu5Gc</td>
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<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
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<tr>
<td>NMP</td>
<td>1-(2-naphthyl)-3-methyl-5-pyrazolone</td>
</tr>
<tr>
<td>NP</td>
<td>Normal phase</td>
</tr>
<tr>
<td>OPD</td>
<td>Ortho-phenylenediamine</td>
</tr>
<tr>
<td>OST</td>
<td>Oligosaccharyltransferase</td>
</tr>
<tr>
<td>PAD</td>
<td>Pulsed amperometric detection</td>
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<tr>
<td>PAEA</td>
<td>2-(2-pyridilamino)ethylamine</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>PGC</td>
<td>Porous graphitized carbon</td>
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<tr>
<td>PMP</td>
<td>1-phenyl-3-methyl-5-pyrazolone</td>
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<tr>
<td>PNGase</td>
<td>Peptide N-glycosidase</td>
</tr>
<tr>
<td>ProA</td>
<td>Procainamide</td>
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<tr>
<td>RF-MS</td>
<td>RapiFluor-MS</td>
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<tr>
<td>RNase B</td>
<td>Bovine pancreatic ribonuclease B</td>
</tr>
<tr>
<td>Rf</td>
<td>Retention factor</td>
</tr>
<tr>
<td>RP</td>
<td>Reversed phase</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RTNT</td>
<td>Retention time</td>
</tr>
<tr>
<td>sDHB</td>
<td>Super-DHB</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SEC</td>
<td>Size exclusion chromatography</td>
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<tr>
<td>SPAAC</td>
<td>Strain promoted azide-alkyne cycloaddition</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TOF</td>
<td>Time of flight</td>
</tr>
<tr>
<td>UDP-GlcUA</td>
<td>Uridine diphosphate glucuronic acid</td>
</tr>
<tr>
<td>UDP-neu5Ac</td>
<td>Uridine diphosphate N-acetyleneuraminic acid</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>UV-Vis</td>
<td>Ultraviolet visible</td>
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<tr>
<td>WAX</td>
<td>Weak anion exchange</td>
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Chapter 1 – Introduction

1.1 Structures of glycans

Biological molecules such as proteins and lipids may be decorated with large and structurally complex carbohydrates \(^1\). These carbohydrates (or glycans) are ubiquitous in nature and are present on proteins of mammals, invertebrates, bacteria, fungi and plants. The process of glycosylation whereby a noncarbohydrate group is modified by the addition of carbohydrate moiety is thought to be the most common form of protein modification with 50% of human proteins estimated to contain a covalently attached sugar moiety at some point in their life cycle \(^2\)-\(^4\). The structure of glycans are diverse. Figure 1 shows the diversity in the structures of common animal glycans \(^5\). Diversity of the glycans is in part due to the variety of different monosaccharides that may be incorporated into the glycan, the functional groups modifications of the monosaccharides, and whether these monosaccharides are linked to produce branched or linear structures.
Figure 1: Common classes of animal glycans and a flowchart depicting types of glycoconjugates and their differences. Figure recreated with permission Cells (Creative Commons License) 6.
1.1.1 Monosaccharides

A monosaccharide is the simplest form of carbohydrate, one that cannot be hydrolysed into smaller carbohydrates. A large number of monosaccharides types can potentially be incorporated into the structure of glycans. Simple hexose monosaccharides all have the same general empirical formula \(C_n(H_2O)_m\), and many differ only in configuration around a single chiral carbon (epimers); the C-2 epimer of D-mannose 2 is D-glucose 1 whereas D-galactose 3 is the C-4 epimer of D-glucose 1, this epimerisation is shown in Figure 2. These simple sugars can be represented in three ways, the Fischer projection, the Haworth projection and the chair conformation. Table 1 shows the structures of the simple hexose in these three forms.

![Figure 2: Monosaccharide Structures showing the epimerization of glucose 1, mannose 2 and galactose 3.](image)

Simple pentoses are a class of monosaccharide that contain five carbon atoms with the general chemical formula \(C_n(H_2O)_m\). Hexose deoxy sugars are another class of monosaccharides. These monosaccharides contain six-membered rings but lack a hydroxyl group on the carbon at the 6-position and have the chemical formula
C₆H₁₂O₅. Fucose is an example of a hexose deoxy sugar and is a common component of both mammalian and fungal glycans.

Table 1: Simple monosaccharide hexoses.

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Fischer projection</th>
<th>Haworth Projection</th>
<th>Chair conformation</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-allose</td>
<td><img src="image1" alt="D-allose Fischer projection" /></td>
<td><img src="image2" alt="D-allose Haworth projection" /></td>
<td><img src="image3" alt="D-allose chair conformation" /></td>
</tr>
<tr>
<td>D-altrose</td>
<td><img src="image4" alt="D-altrose Fischer projection" /></td>
<td><img src="image5" alt="D-altrose Haworth projection" /></td>
<td><img src="image6" alt="D-altrose chair conformation" /></td>
</tr>
<tr>
<td>D-glucose</td>
<td><img src="image7" alt="D-glucose Fischer projection" /></td>
<td><img src="image8" alt="D-glucose Haworth projection" /></td>
<td><img src="image9" alt="D-glucose chair conformation" /></td>
</tr>
<tr>
<td>D-mannose</td>
<td><img src="image10" alt="D-mannose Fischer projection" /></td>
<td><img src="image11" alt="D-mannose Haworth projection" /></td>
<td><img src="image12" alt="D-mannose chair conformation" /></td>
</tr>
<tr>
<td>D-gulose</td>
<td><img src="image13" alt="D-gulose Fischer projection" /></td>
<td><img src="image14" alt="D-gulose Haworth projection" /></td>
<td><img src="image15" alt="D-gulose chair conformation" /></td>
</tr>
</tbody>
</table>
Glycans may also incorporate derivatised monosaccharides. These include phosphorylated monosaccharides, such as D-glucose-1-phosphate 4, aminated monosaccharides such as D-glucosamine 5 and acetylated amines such as D-N-acetylglucosamine (GlcNAc) 6 (Figure 3).

**Figure 3:** Structures of D-glucose-1-phosphate 4, D-glucosamine 5 and GlcNAc 6.

Glycans may also be composed of uronic acids, these are a class of sugar acid formed via oxidation of the C-6 hydroxyl group of an aldose. Other acidic monosaccharides include sialic acids which also feature in glycans. Sialic acids are a
class of nine-carbon acidic sugars with highly variable structures commonly found at the terminal non-reducing ends of glycans \(^8,9\). The reducing end of the glycan contains an aldehyde or a ketone group which may be reduced to form a glycosidic bond whereas the non-reducing end does not contain any groups that could be reduced. Variation in sialic acids arises from the modifications that may occur on one or more of hydroxyl groups of the parent structure neuraminic acid 7. Common modifications include acetylation, methylation and sulfation \(^10\). The most abundant sialic acid on mammalian glycans is \(N\)-acetylneuraminic acid 8 (Neu5Ac), the acetyl derivative of neuraminic acid 5. Variations in sialic acid structure are dependent on the species they are produced in as sialic acid present in lower mammals, such as \(N\)-glycolylneuraminic acid 9 (Neu5Gc), is not naturally occurring in humans (Figure 4) \(^11\). This is due to a 92-bp frame shifting exon deletion approximately two to three millions years ago which resulted in the loss of activity for the cytidine monophosphate-\(N\)-acetylneuraminic acid hydroxylase enzyme in primates and humans \(^12\).

![Chemical structures](image)

**Figure 4:** Structures of neuraminic acid 7, Neu5Ac 8 and Neu5Gc 9.

These modifications mean that over eighty different structures of sialic acids have been observed. **Figure 5** shows the variety of possible linkages behind the diversity of this class of monosaccharide.
Figure 5: Diversity of sialic acids\textsuperscript{13}.

1.1.2 Glycosidic linkages

Glycosidic linkages between the monosaccharides contribute to the variation that is observed in glycans. Glycosidic bonds may exist with $\alpha$ or $\beta$ stereochemistry depending on the position of the glycosidic oxygen above or below the sugar ring. Glycosidic bonds may exist at several position of each monosaccharide ring producing variety in connection and branching. For example three different hexoses could be assembled theoretically to produce $27,648$ unique trisaccharides\textsuperscript{14,15}. \textbf{Figure 6} shows the structural variation that can occur from different glycosidic bonds between glucose and lactose.
Figure 6: Structures of glucose differently glycosidically linked to lactose.

1.1.3 Glycoconjugates

Variation is also seen through the linkages that oligosaccharides form between other types of chemical constituents. These are known under the general name of glycoconjugates. Examples of glycoconjugates include $N$-linked glycans, $O$-linked glycans, $O$-linked glycosaminoglycans (GAGs) of proteoglycans, glycopeptides, peptidoglycans, glycolipids and lipopolysaccharides. These glycoconjugates are discussed in more detail in the following sections.
1.1.3.1 N-linked glycans

N-glycosylation is a co-translational modification. Glycans are added to the nascent polypeptide chain as it is synthesised by oligosaccharyl transferase (OST). The enzyme transfers assembled oligosaccharides from the lipid linked dolichol precursor to selected asparagine residues of polypeptides as they enter the lumen of the endoplasmic reticulum (ER) $^{16}$. Lipid linked dolichol-GlcNAc-Man5 is constructed on the cytoplasmic side of the ER shown in steps I to III of Figure 7 and is then translocated into the lumenal side of the ER by a class of enzymes called flippases. Further mannose are added by dolichol phosphate mannose donors then glucose is added from dolichol phosphate glucose donors in steps IV and V of Figure 7 $^{17}$. The addition of the glucose cap is used as a quality control process in the biosynthesis of the dolichol linked precursor. These precursors are transferred onto the nascent polypeptide in the lumen of the ER. The glucose residues are instrumental in the chaperone assisted proofreading of protein folding known as the calnexin/calreticulin cycle. If the structure is correct the glucose residues are removed and the glycan can be processed by a series of mannosidases $^{18-20}$.

Figure 7: Biosynthesis of the dolichol oligosaccharide precursor. First UDP-GlcNAc donors add GlcNAc 6 to dolichol phosphate by GlcNAc-1-phosphotransferase and by GlcNAc transferase in step two. In step three mannose is added by GDP-Man donors. The 5-
A mannose product is then flipped to the lumen side of the ER. Here a further four mannoses are added by Dol-P-Man donors in step four. The final dolichol oligosaccharide precursor is completed by addition of three glucose from Dol-P-Glc donors. This final product can then be transferred to asparagine.

*N*-linked glycans all share a consistent Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAcβ1-Asn-X-Thr/Ser core sequence due to the transfer of the dolichol oligosaccharide precursor. The rest of the glycan shows variation in the monosaccharides present, the antennae that are attached and the degree of bisection. Even with these variations *N*-linked glycans can be classified into one of three types of structures: the high mannose type, the hybrid type and complex type. These three structures are each shown in Figure 8.

**Figure 8:** Examples of the three structures of an *N*-linked glycan. The condition of the line indicates the type of glycosidic linkage, the direction of the bond indicates the linkage between the two monosaccharides and the shape and the colour of the shape indicates the monosaccharide that is incorporated in the glycan.
Figure 8 (a) is an oligomannose glycan, this type of glycans is composed of only mannose residues attached to the core. Figure 8 (b) is a complex glycan, these structures have antennae initiated by \(N\)-acetylglucosaminyltransferase attached to the core \(^{22}\). Finally, Figure 8 (c) is a hybrid glycan, these have antennae initiated by \(N\)-acetylglucosaminyltransferase attached to the Man-\(\alpha1,6\) arm of the core and one or two antennae composed of only mannose on the Man-\(\alpha1,3\) arm \(^{22}\).

\(N\)-linked glycans are found attached to proteins via asparagine residues at specific sequons (Asn-\(X\)-Ser or Asn-\(X\)-Thr where \(X\) is any amino acid except proline) \(^{3}\). Although glycans are only found at these sequons, not all sequons will bear glycosylation. Rarely a consensus \(N\)-link can be made at Asn-\(X\)-Cys residue \(^{24}\). An example of the glycosidic linkage between the reducing end GlcNAc 6 of a glycan with each of these sequons is shown in Figure 9.

![Figure 9: Structure of an Asn-X-Ser (left) and Asn-X-Thr (right) N-Glycan linkage. Asn is shown in red, the X amino acid is shown in blue, Ser is shown in purple and Thr is green. The glycan is linked to the amine group of Asn with the \(R_1\) group representing the rest of the glycan.](image)

The inability of proline to form part of the sequon is thought to be due to the orientation necessary for the peptide chain to bind in the enzyme active site. The ring constrained amine in proline limits the orientation that the amino acid sequence can undertake. Crystal structures of bacterial OST have shown the active
site requires that the amino acid sequence adopts an 180° orientation which proline is not capable of (Figure 10) 25.

Figure 10: The 180° orientation of the protein backbone in the bacterial OST active site. a) Transmembrane and periplasmic domains of bacterial OST are in blue and green. Acceptor peptide is in ball and stick representation; N and C denote amino and carboxy termini. Peptide residues are yellow and acceptor Asn is labelled red. b) shows the chemical structure of bacterial lipid linked oligosaccharide in white, the orientation between the two domains results in the nucleophilic attack from asparagine amine to the lipid linked oligosaccharide. Reprinted with permission from Nature 26.

$N$-linked glycan possess a variety of function including protein folding, regulation and degradation, and transport and targeting 27. Previous studies have indicated that the presence of appropriate $N$-linked glycans modulate integrin activation. Integrins are the principal receptors used by animal cells to bind to extracellular matrix (ECM) 28. Fibroblast cells cultured in l-deoxymannojirimycin, an inhibitor of $N$-linked glycan processing, resulted in the presentation of immature integrin $\alpha5\beta1$ at the cell surface 29. These integrins led to greatly reduced cell adhesion between the fibroblast and the ECM. The treatment of purified integrin $\alpha5\beta1$ with $N$-glycosidase F (PNGase F) which removes the glycan at the innermost GlcNAc residue resulted in blockage of integrin $\alpha5\beta1$ binding to the ECM. These observation indicate that $N$-linked glycosylation is essential for functional adhesion to integrin $\alpha5\beta1$ 29.

1.1.3.2 $O$-linked glycans
Glycans may also be attached to the protein backbone through a glycosidic bond between a monosaccharide and the hydroxyl group of serine or threonine residues. It is unknown if there is a consensus sequence for this attachment. Glycans linked in this manner are known as O-linked glycans and are a common covalent modification of serine and threonine residues in mammalian glycoproteins. Potential monosaccharides include N-acetylgalactosamine (GalNAc), GlcNAc, mannose, galactose, fucose and glucose. The glycosidic linkage between GalNAc with serine and threonine is shown in Figure 11.

![Figure 11: Structure of the O-linked glycosidic bond between GalNAc and serine (left) and threonine (right). R₁ represents the rest of the glycan.](image)

Mucins are family of heavily O-glycosylated proteins produced by epithelial cells in animals. Mucins carry the greatest number of O-GalNAc glycans, also known as mucin-type O-glycans. O-GalNAc glycosylation unlike N-glycosylation requires no lipid linked intermediate. These glycans play various roles on mucins. The O-GalNAc glycan are ubiquitous on mucins and effect the conformation of the protein, inducing the protein to adopt a stiff structure, preventing folding into a globular structure. The O-GalNAc glycan on mucins are essential to hydrate and protect underlying epithelium as they bind water to the surface of the epithelial cells which is thought to protect the mucosa from dehydration. The O-glycans on mucins are also responsible for trapping bacteria as they contribute to the trapping properties of mucous. The structure of the core mucin O-glycans are shown in Figure 12.
1.1.3.3 Proteoglycans

Proteoglycans are another example of a glycan linked through an O-glycosidic bond. This glycoconjugate is an example of a glycosaminoglycan (GAG), a term used to describe hexosamine containing polysaccharides. Proteoglycans consist of a core protein portion with one or more GAGs covalently attached to Ser in a Ser-Gly sequence in the protein backbone by a xylose residue in the tetrasaccharide linker composed of glucuronic acid–galactose–galactose–xylose. These GAGs are linear polysaccharides composed of repeating disaccharide units of an acetomido sugar such as GlcNAc, or GalNAc, or amino sugar such as glucosamine or galactosamine with galactose or an uronic acid such as glucuronic acid or iduronic acid. The amino sugar or acetomido sugar may also be sulfated in the case of chondroitin sulphate, dermatan sulfate and heparan sulfate.

The major classes of GAGs that decorate proteoglycans include, chondroitin sulphate, dermatan sulphate, heparan sulphate and heparin. The structure of heparan sulfate is shown in Figure 13. GAGs present on proteoglycans are similar...
between vertebrates and invertebrates, this is unlike glycans which display large differences between species which shows variation in monosaccharides and structure. The GAGs on proteoglycans are also generally larger than N- and O-linked glycans with GAGs containing 80 sugar residues and masses of 20 kDa compared to the 10-12 monosaccharide residues seen for N-linked glycans. Proteoglycans display high structural variability due to the large number of potential chain lengths, saccharide sequences, domain arrangements, substitutions and compositions, as well as the modifications that occur such as sulfonation.

Figure 13: Structure of heparan sulfate.

Adding to the variation is the protein core expression level and GAGylation, proteins may be attached to one or two types of GAGs with this varying between cell type. Structural variability also stems from the quantity of GAGs present, proteoglycans may only contain one GAG, while others contain more than 100. The production of proteoglycans occurs within virtually all metazoan cells. The protein is synthesized in ribosomes and translocated into the lumen of the rough ER. The glycosylation of the protein is performed enzymatically in the Golgi apparatus. The linking tetrasaccharide is attached to the serine side chain which acts as a primer for polysaccharide growth. Monosaccharides are added one at a time by glycosyl transferases except for heparan sulfate where elongation of the chain is performed by two polymerases, exostosin-1 and exostosin-2. The particular glycosyltransferases will differ depending on the GAG that is being produced, the most common proteoglycan, chondroitin sulphate is made from...
disaccharide units of glucuronic acid and GalNAc by glucuronic acid transferase II and N-acetylgalactosamine transferase II. The completed proteoglycan is then exported in a secretory vesicle before secretion onto plasma membranes or into the ECM.

Proteoglycans display various roles including cell surface receptors and signalling modulators. For example, the adhesion of erythrocytes infected by malaria to endothelial cells requires chondroitin sulphate chains. Proteoglycans in the ECM also promote cell adhesion by facilitating attachment of cells to ECM components. Heparan sulphate proteoglycans are necessary to form adhesion sites with fibronectin, a protein a high mass protein that binds to membrane receptors integrins. GAGs may also act as coreceptors. A coreceptor is a cell surface molecule that influences ligand-receptor activity but does not contain intrinsic catalytic activity. Heparan sulfate, a polysaccharide chain containing repeating units of glucuronic acid and GlcNAc as a coreceptor of fibroblast growth factor receptors. The fibroblast growth factor receptors consist of two or three extracellular immunoglobulin linked by a transmembrane region to a cytoplasmic domain. The phosphorylation of the cytoplasmic domain of the fibroblast growth factor receptor becomes phosphorylated following the binding with the receptor and the coreceptor heparan sulfate. This phosphorylation leads to downstream signalling leading to normal developmental processes such as tissue repair.

1.1.3.4 Hyaluronan

Hyaluronan is a linear polysaccharide composed of repeating GlcNAc-β1,4-GlcUA-β1,3 forming a large polymer that may be 10,000 disaccharides long and have a mass of approximately 4 million daltons (Da). Hyaluronan is also a GAG conserved throughout all mammals.
Figure 14: Repeating unit of hyaluronan.

The synthesis of hyaluronan is tightly regulated due to the large amount of adenosine triphosphate that is required \(^{53}\). Commonly, GAGs are synthesised in the cells Golgi network, however hyaluronan is synthesized in the inner face of the cellular plasma membrane as a free polymer \(^{54}\). Hyaluronan is synthesised by three hyaluronan synthase isoenzyme (HAS1, HAS2 and HAS3) with the substrates uridine diphosphate N-acetylneuraminic acid (UDP-Neu5Ac) and uridine diphosphate glucuronic acid (UDP-GlcUA) \(^{55, 56}\). Hyaluronan is found predominantly in the ECM or bound to the CD44 receptor at the cell surface and introduces elastoviscosity of liquid connective tissues such as joint synovial fluid and eye vitreous fluid \(^{57, 58}\). One of its functions in the body is to bind water and lubricate movable parts of the body such as muscles and joints \(^{57, 59}\). Under physiological conditions, hyaluronan is stiffened by internal hydrogen bonds \(^{37}\). The axial hydrogens atoms form a non-polar face while the equatorial side chains form a polar face \(^{37}\). This causes the formation of a twisting helical configuration, meaning hyaluronan can trap 1000 times its weight in water \(^{37}\).

Hyaluronan also form roles in protein adhesion due to the ECM due to interaction of hyaluronan with CD44 receptors \(^{60}\). The CD44 receptor is regarded as the principal receptor for hyaluronan. CD44 is a multifunctional single pass transmembrane glycoprotein or proteoglycan consisting of an amino terminal domain, stem structure, transmembrane domain and cytoplasmic domain \(^{61}\). The
amino terminal domain contains motifs that provide docking sites for ECM components such as hyaluronan supporting adhesion for cells displaying CD44 \(^6\).  

1.1.3.5 Glycosylphosphatidylinositol linked anchor

Another glycoconjugate present at either termini of proteins in nature are glycosylphosphatidylinositol (GPI) structures, also known as GPI anchors \(^6\). These structures are ubiquitous in eukaryotes and have been identified in virtually all eukaryotes ranging from fungi to humans \(^6\). Over 150 GPI-anchored proteins have been observed in humans with a variety of functions \(^6\).

Mature GPI anchors show high structural variability but share a common structural core composed of Man-\(\alpha_1,2\)-Man-\(\alpha_1,6\)-Man-\(\alpha_1,4\)-GlcNAc-\(\alpha_1,6\)-myo-inositol which is a universal hallmark of GPI anchors \(^6\). The biosynthesis of GPI anchors occurs in three stages, first the GPI phosphatidylinositol lipid precursor is assembled enzymatically in the ER. In the second stage of biosynthesis, the precursor is covalently attached to the carboxyl terminus of a newly synthesized protein via an ethanolamine phosphate bridge, between the C-6 of the monosaccharide, and an \(\alpha\)-carboxyl group of the amino acid. Finally, in the third stage, lipid remodelling and side chain modification occurs in the ER \(^6\). After modification the result is a mature GPI anchors which displays diverse structures, with diversity as a result of the organism the GPI anchor is synthesised in and the protein to which they are attached \(^6\). The structure of a GPI anchored protein is shown in Figure 15.
The N-linked glycans of GPI anchor proteins are required for the efficient trafficking of proteins to the Golgi as the glycans function as ER exit signals. This was demonstrated by Fujita et al. (2009), that observed that the transport of GPls from the ER relied on the enzyme PGAP5. PGAP5 enzymatically removes the side chain EtNP on Man$_2$ of GPI anchors suggesting that the processing of the GPI glycan is critical for the transport of the GPI protein from the ER to the Golgi.

1.1.3.6 Glycolipids

The final glycoconjugate mentioned in this section is the glycolipid. This term is applied to any monosaccharide or oligosaccharide glycosidically linked to a hydrophobic moiety such as acylglycerol, sphingoid, ceramide or phenyl phosphate. When mono-, di- or trisaccharides are glycosidically linked to a diglyceride they are classified as glycoglycerolipids (Figure 16), and glycosphingolipids when monosaccharides are linked to ceramide moieties, which are amides of fatty acids. Glycosphingolipids include oligoglycosylceramides, neutral glycosphingolipids and acidic glycosphingolipids such as sulfosphingolipids and gangliosides.
Figure 16: The structure of a glycoglycerolipid.

Glycolipids are found widely throughout eukaryotic cells in all kingdoms but are common features of cellular membranes in mammalian cells\textsuperscript{67-69}. The variety of glycolipid is attributed to the combination of glycosyltransferase found in different species and cell types. The biosynthesis of the ceramide glycolipid starts with the biosynthesis of ceramide which occurs on the cystolic face of the ER before it is transported to the Golgi\textsuperscript{70}. This ceramide is then used in the synthesis of glucosylceramide-derived glycolipids by the addition of glucose with glycosyltransferases. The glucosylceramide formed at the cytoplasmic surface of the \textit{cis} Golgi is then translocated along the Golgi stack to the luminal surface of the membrane. This substrate is then used by other subsequent glycosyltransferases to build a higher order neutral glycolipid. The formation of some higher order ceramide glycolipids and the transferases that undertake this biosynthesis is shown in Figure 17.
Figure 17: Schematic representation of the biosynthesis of sphingolipids. Glc-T, ceramide glucosyltransferase; GalNAc-T, N-acetylgalactosaminyltransferase; Gal-T, ceramide galactosyltransferase; Sial-T4, GM1 sialyltransferase; Sial-T1, lactosylceramide sialyltransferase; Sial-T2, GM3 sialyltransferase.

The glycolipids on cellular membranes play not only a structural role in maintaining membrane stability by forming hydrogen bonds with the surrounding water but glycolipids also act as receptors to facilitate cell-cell communication acting as receptors, anchors for proteins and regulators of signal transduction. The glycolipid GD3 is highly expressed in melanomas cells and plays roles in adhesion of these cells to the ECM. An investigation on the effect of GD3 adhesion signals to augment the malignant properties of melanoma cells by Ohkawa et al. (2010) examined the adhesion of GD3+ and GD3- cells to the ECM. These results suggested that strong cells adhesion was induced by GD3 expression which caused increased interaction between integrins, This suggests that GD3 is implicated in integrin mediated cell adhesion.

The modulation of signal transduction is caused by the formation of glycolipids microdomains in the lipid bilayer. These glycolipid clusters are known to associate with acylated proteins like cSrc, Src family kinases and focal adhesion kinase.
Kinases are modified by saturated chain-lipids which insert preferentially from the glycolipid microdomains. The stimulation of the glycolipid microdomain induces conformational changes to the associated protein which triggers signal transduction.

1.2 Importance of glycans in biotherapeutics

The glycans mentioned in the previous section have highlighted the functions of these molecules play in cell adhesion, cell signalling and elastoviscosity. Glycans on biopharmaceuticals have been shown to have a profound influence on the serum half-life and safety of therapeutic proteins. Many biopharmaceuticals are produced in mammalian cells, such as Chinese hamster ovary cells (CHO), baby hamster kidney cells (BHK) and mouse myeloma cells. The glycans displayed on the biopharmaceuticals may differ depending on the culture conditions and may exhibit batch-to-batch variation. These are a response to minute changes in environmental conditions including nutrient levels, dissolved oxygen levels, pH, temperature, stress, the use of serum and growth on microcarriers. Even well-controlled biologic production may result in a heterogeneous mixture of glycoforms.

CHO cell lines dominate the commercial production of biologics accounting for over 70% of approved biotherapeutics including adalimumab (Humira, Abbvie), trastuzumab (Herceptin, Genentech) and Omalizumab (Xolair, Genentech). CHO cells are used so frequently due to the well-established regulatory track record, good safety profile and ease of handling. CHO cells are tolerant to changes in culture conditions including pH, oxygen level pressure and temperature. Biopharmaceuticals produced by CHO cells exhibit ‘human like’ N-glycosylation patterns. This meaning they show glycans with similar structures and with similar monosaccharides especially when compared to other possible host system such as
bacteria or plant systems which show greater variation in monosaccharides than mammals\(^97\). However, even though greater similarity is seen with CHO cells over other host systems, CHO cells produce glycosylation patterns in N-glycans that are not produced by human cells featuring \(\alpha-2,3\) and \(\alpha-2,6\)-linked sialylation. CHO cells predominately exhibit \(\alpha-2,3\)-linked sialic acids as CHO cells lack the \(\alpha-2,6\) sialyltransferase gene\(^98\). Further differences in the biosynthetic sialylation pathways of CHO cells mean that biopharmaceutical glycans produced in CHO cells may feature terminal sialic acids structures featuring Neu5Gc\(^9\) which is not naturally produced by humans\(^99,100\).

1.2.1 Immunogenicity of Neu5Gc\(^9\) containing glycans

Some features of glycans are known to cause immunogenic response in patients\(^101\). The features responsible are typically non-human and are found on biologics that have been produced in mammalian cells\(^102\). Neu5Gc\(^9\) (also known as Hanganutziu-Deicher antigen) is an immunogenic monosaccharide produced enzymatically by the transformation of Neu5Ac\(^8\) by CMP-\(N\)-acetylneuraminic acid hydroxylase (CMAH). The transformation is shown Figure 18 through the conversion of the 5-acetyl group to the 5-glycolyl group\(^103\).

![Figure 18: Transformation of Neu5Ac 8 to Neu5Gc 9.](image)

Although common in many lower mammals, the enzyme responsible for Neu5Gc\(^9\) biosynthesis is mutated in humans so Neu5Gc\(^9\) is not naturally seen in human
glycans. As biopharmaceuticals are produced in the cell lines of lower mammals which express CMAH, glycoproteins synthesised in these host cells display small amounts of Neu5Gc ⁹⁴. For example human recombinant erythropoietin (hRuEPO) produced in CHO cells routinely have a Neu5Gc ⁹ content of around 1% of total sialic acid content ⁹⁵. Neu5Gc ⁹ causes immunogenic responses in humans by binding with the antibody anti-Neu5Gc. This forms an immune complex which causes inflammation and potentially anaphylaxis ⁹³. High levels of Neu5Gc ⁹ also results in increased elimination of the biologic from the body as it is sequestered by the formation of the immunocomplex ⁹¹.

1.2.2 Immunogenicity of alpha gal containing glycans

Other immunogenic non-human features present on glycans produced in mammalian cell lines are glycans with the galactose-α1,3-galactose epitope. This epitope is catalysed by α-1,3-galactosyltransferase, which is inactive in humans and higher primates but present in lower mammals. This galactose-α1,3-galactose epitope also known as alpha gal is implicated in anaphylaxis.

Cetuximab® is a chimeric mouse monoclonal antibody (mAbs) used in the treatment and management of metastatic colorectal cancer, non-small cell lung cancer and head and neck cancers by inhibiting the epidermal growth factor. Cetuximab has been associated with producing hypersensitivity reactions due to the carbohydrate structures displayed on the surface of the protein ⁹⁶ Cetuximab® is produced in mouse myeloma cells which have the biosynthetic capacity to produce glycans featuring alpha gal ⁹⁷. A total of twenty-one oligosaccharides have been characterised from Cetuximab®, 30% of which display the galactose-α-1,3-galactose glycosidic linkage shown in Figure 19.
Biopharmaceuticals such as antibodies, may be degraded by non-specific proteolytic cleavage\textsuperscript{108}. This unintended proteolysis is a result of the peptidase and proteinase repertoire of circulating phagocytic cells\textsuperscript{109,110}. Changes to the glycosylation of biopharmaceuticals, for example the variations in sialic acids, often result in changes in drug serum lifetimes\textsuperscript{78,80}. This occurs as sialic acids mask the glycoprotein, significantly increasing the serum half-life of biologics with glycans featuring terminal sialic acids. Glycans devoid of terminal sialic acids residues are removed faster from circulation due to the exposed galactose residues interacting with the asialoglycoprotein receptors on hepatocytes which leads to their elimination for the body\textsuperscript{111-113}. These receptors bind non-sialylated glycoproteins and remove them from the serum by endocytosis\textsuperscript{113}.

This effect on serum half-life is seen with hRuEPO. Erythropoietin (EPO) is a glycoprotein synthesised naturally in kidney peritubular fibroblasts and regulates erythropoiesis (red blood corpuscle production) and its recombinant version is used to treat anaemia\textsuperscript{114,115}. The interaction of EPO with an EPO-receptor leads to a cell signalling activation pathway which increases the biosynthesis of haemoglobin by causing haematopoietic stem cells in bone marrow to differentiate into mature blood cells\textsuperscript{116}. hRuEPO consists of 145 amino acids and has a mass between 30-34 kDa. The amino acid chain is heavily glycosylated, with 40% of the mass of the protein attributable to the carbohydrate modifications\textsuperscript{117}. The protein backbone
contains three possible modifiable asparagine sequons at positions 24, 38 and 83, where N-linked glycans may be found. Each position has the potential of hosting a tetraantennary glycan each with the potential of hosting four sialic acid residues. A further two possible sialic acid residues can be accommodated on an O-linked glycan on serine at position 126 for a total possible 14 sialic acid residues. hRuEPO containing a total 14 sialic acid residues resulted in an approximate three-fold increase in serum half-life compared to native EPO due to preventing the removal of the non-sialylated glycoprotein by hepatic asialoglycoprotein receptors. Increased serum half-life was also noticed when the terminal monosaccharides of a glycan was mannose or GlcNAc.

1.3 The analysis of glycans

The structure of glycans are commonly analysed in industrial and research settings. Analysis of glycans on biopharmaceuticals is a standard regulatory procedure in biopharmaceutical production. One route of analysis commonly starts with release of glycans, following release, glycans may be derivatised to facilitate analysis then purified and analysed. However, workflows differ and some methods do not require all of these processes.

The release of the glycans simplifies the analysis of the glycan of interest as the presence of the protein complicates analysis. Various methods are employed to liberate the glycan from the protein chain and the method used relies on the type of glycoconjugate that the glycans is being released from. However, release methods fall into two categories. These categories are release by chemical means, where a chemical reaction breaks the glycosidic bond, or enzymatically where an enzyme does the same. Some of the methods used in the release of glycans are detailed in the following sections.
1.3.1 Glycan analysis workflows

The structural analysis of glycans is commonly performed in research and industrial settings. The analysis of glycans on biopharmaceuticals is a standard regulatory procedure in biopharmaceutical production\textsuperscript{121}. Releasing the glycan simplifies the analysis as the presence of the protein backbone introduces complications. Various methods are employed to liberate the glycan from the protein chain depending on the glycoconjugate.

1.3.1.1 Chemical release of glycans

The chemical means of glycan removal that are used have a few criteria. The method should be non-selective in the separation of the glycan and should not cause modifications to the glycan. The release should also ensure sufficient recovery of the glycan to make further analysis possible. The commonly used methods of chemical release are β-elimination and hydrazinolysis. These methods were first developed for the release of \textit{N}-linked glycans by Takasaki et al (1983) and are now applied to the release of other glycoconjugates including \textit{O}-linked glycans, GPI anchors and proteoglycans\textsuperscript{122-124}.

1.3.1.1.1 Alkaline β-elimination of glycans

The release of glycans from an \textit{O}-glycosidic linkage can take place by β-elimination under mild alkaline conditions. This method is the most common chemical method of \textit{O}-glycan removal\textsuperscript{125}. \textit{O}-glycosidic linkages are readily hydrolyzed by dilute alkaline solutions (0.05M to 0.1M solutions of either sodium hydroxide or potassium hydroxide) under mild conditions (45 - 60 °C, 8 - 16 h)\textsuperscript{126}. This reaction must be performed under reducing conditions by using reducing agents (0.8M to 2M sodium borohydride) to prevent the loss of a monosaccharide from the
reducing terminus of the glycans also known as peeling\textsuperscript{124}. This reaction is shown in Scheme 1 and forms the reduced alditol form of the glycan and causes the deacetylation of any GlcNAc 6 residue in the sample which must be reacetylated with acetic anhydride or reacted further as the alditol form.

Scheme 1: Alkaline $\beta$-elimination of an O-glycan.

This method has issues associated with the use of sodium hydroxide and sodium borohydride as the sodium content remaining after the reaction interferes downstream with some detection methods such as mass spectrometry (MS)\textsuperscript{126, 127}. Developments on the method of $\beta$-elimination have been developed to reduce high sodium content by using ammonia instead of sodium hydroxide under non-reducing conditions. However, with this development, glycan liberation is incomplete for some linkages and peeling was not entirely prevented\textsuperscript{128, 129}. $N$-linked glycans release by $\beta$-elimination requires harsher alkaline conditions for effective release (1M sodium hydroxide at 100 °C for 6 to 12 h). For this reason removal of these glycans by this method is not common\textsuperscript{130}.

1.3.1.1.2 Hydrazinolysis $\beta$-elimination of glycans

Hydrazinolysis is another form of $\beta$-elimination, this technique of chemical glycan release uses hydrazine to cleave the glycans from the peptide\textsuperscript{124}. This process is shown in the hydrazinolysis step in Scheme 2. The release from the protein results in the formation of a hydrazine on the reducing end of the glycan. The hydrazine product is acetylated in the re-$N$-acetylation arrow and hydrolyzed to a hydroxyl group with TFA in the restoration of reducing terminus step. This step eliminates
the acetyl group on the reducing end but can cause loss of structural information such as the loss of acid labile groups.\textsuperscript{131}

\begin{center}
\includegraphics[width=\textwidth]{scheme2.png}
\end{center}

\textbf{Scheme 2: Glycan release by hydrazinolysis elimination.}

1.3.1.2 Enzymatic release of glycans

The other common method of glycan release is performed with enzymes.\textsuperscript{132} The growing group of enzymes capable of releasing glycans such as endoglycosidases and glycosamidases has provided a method for glycan removal under mild conditions.\textsuperscript{133} This enzymatic removal can be performed for both N-linked and O-linked glycoproteins, proteoglycans, GPI anchors and glycolipids.\textsuperscript{134, 135} However, some limitations are seen in the removal of glycans when enzymes lack specificity or when enzymes show restricted specificity to the substrate which limits enzymatic release for some types of glycans.\textsuperscript{133}

The removal of N-linked glycans is performed reliably enzymatically with peptide N-glycosidase A (PNGaseA) and peptide N-glycosidase F (PNGaseF).\textsuperscript{136} PNGases are effective methods of releasing N-linked glycans and are widely used due to the ability for them to remove almost every N-linked oligosaccharide and show very little activity for other carbohydrate residues.\textsuperscript{137} Both PNGaseA and PNGaseF
cleave between the innermost GlcNAc 6 and asparagine on glycoproteins (Figure 20). PNGaseF is active against most N-linked glycans but is not active against glycans containing a GlcNAc-α1,3-fucose link at the core. PNGaseF is active against most N-linked glycans but is not active against glycans containing a GlcNAc-α1,3-fucose link at the core.

Figure 20: Cleavage site of PNGase F. R₁ represents the remaining portion of the glycan. R₂ and R₃ represents the rest of the protein backbone.

The removal of O-linked glycans enzymatically pose a greater challenge due to the lack of an O-linked equivalent to PNGase: a single enzyme which reliably releases the intact O-linked structures. This means the release of the intact O-linked glycan is not possible preventing the analysis of the intact glycan. O-glycosidases can remove the O-linked glycans from the protein backbone but is only active towards the Gal-β1,3-GalNAc core. This means that the monosaccharides are first removed sequentially by exoglycosidases until the core Gal-β1,3-GalNAc remains which can then be removed. Other modifications present on the glycan, sialylation for example, prevents the action of exoglycosidases and these monosaccharides must first be removed enzymatically by neuraminidases.

1.3.1.3 Derivatization of glycans to facilitate analysis
The final process in the analysis of glycans are their detection. This provides the ability to characterize the glycans and determine their structure, however, some methods of detection are incompatible with native glycans for example fluorescence detection (FLD), which requires the presence of a fluorophore that glycans lack. As no group is present on a native glycan that may allow sensitive detection of a native glycan, derivatisation of the glycan may be performed after release from the protein. The addition of a group or molecule that aids the detection of the glycan benefit from groups that are not present in glycans. Some methods of derivatization introduce a fluorophore or increase the hydrophobicity of a glycan to improve detection. Some of the common derivatisation techniques are presented in the following sections.

1.3.1.3.1 Permethylation of glycans

Permethylation is the addition of methyl groups onto the heteroatoms of a glycan. An example of permethylation is shown on the trisaccharide in Figure 21. The permethylation reaction can be performed with iodomethane and sodium hydroxide in dimethyl sulfoxide (DMSO).
Fig. 21: Structure of natural and permethylated trisaccharide.

Permethylation improves the hydrophobicity of the molecule which benefits analysis by MS which shows a hydrophobic bias. Zhou et al (2016) analysed glycans enzymatically released from bovine fetuin which had been derivatised by permethylation. In this analysis, the permethylated samples were found to result in a two-fold increase in ionization efficiency compared to native glycans and also stabilised sialylated glycans in positive mode MS.

1.3.1.3.2 Glycan labelling to facilitate glycan analysis

Glycan ‘labelling’ is another widely used method of derivatisation. This is the addition of a label, a molecule that possesses properties not naturally present in glycans to facilitate analysis. In some cases the analyses detects the label rather than carbohydrate. Labels commonly feature conjugated, hydrophobic and ionisable groups. A variety of potential methods for label addition can be used with
a variety of potential labels, each displaying differing analytical niches which may be employed depending on the chosen method of detection.

1.3.1.3.2.1 Reductive amination labelling of glycans

The first method of label addition covered in this thesis is via reductive amination. Labelling by reduction amination converts the carbonyl group on the reducing end of the glycan \(^{142}\). This reaction forms an intermediate imine before forming a secondary amine with the label via a reducing agent \(^{142}\). The reductive amination reaction is performed in a stochiometric fashion with a single label per glycan \(^{143}\). Labelling is performed with a large excess of both label and reducing agent to ensure high labelling efficiencies \(^{144}\). This reaction is commonly performed in DMSO containing acetic acid but labelling also may be performed in other solvents including tetrahydrofuran (THF) and methanol (MeOH) which are used as alternatives if low pH conditions may result in desialylation \(^{143}\). Acidic conditions are used as the transimination of aniline derivatives is accelerated under acidic conditions \(^{145}\). The commonly used reducing agents include sodium borohydride, sodium cyanoborohydride and picoline borane \(^{143}\). An example of the reductive amination of a glycan with 2-aminobenzamide (2-AB) 10 is shown in Scheme 3.

![Scheme 3: Reductive amination labelling of a glycan with 2-AB 10. R₁ represents the rest of the glycan.](image-url)
1.3.1.3.2.2 Hydrazine labelling of glycans

Labelling with hydrazines or hydrazides is a common method of derivatisation. In this method, a hydrazine or hydrazide molecule reacts with the reducing end of the glycan producing the hydrazone product. The product of this labelling method is shown in Scheme 4. This reaction is performed with a mixture of MeOH and acetic acid at a temperature between 60 – 80 °C. The labelling of carbohydrates including lactose and ovalbumin glycans with phenylhydrazine 11 was performed by Lattova and Perrault (2013). In this research the quantitative derivatisation of lactose was performed at 70 °C for 1 h resulting in a 15-fold increase in detection sensitivity in MS compared to underivatized lactose.

![Scheme 4: Example of a hydrazine labelling reaction with phenylhydrazine 11 on the reducing end of GlcNAc 6 residue. R1 represents the rest of the glycan.](image)

1.3.1.3.2.3 Michael addition labelling of glycans

Michael addition is another method of labelling at the reducing end of glycans. Michael addition is performed under alkaline conditions which prevents the risk of desialylation under acidic conditions and can be performed as a combination chemical release and labelling method. A Michael addition reaction is a
nucleophilic addition of a carbo anion to an unsaturated α, β-unsaturated carbonyl compound \(^{143}\). This labelling reaction is base catalyzed and the addition of the label is a two-step process in which the labelling reagents are consecutively added to the reducing end of the glycan in a two to one stoichiometry \(^{143}\). This labelling therefore results in the addition of two labels per glycan. The Michael addition reaction can be performed with the label 1-phenyl-3-methyl-5-pyrazolone (PMP) \(12\) at 70 °C for 30 minutes in aqueous MeOH with ammonia \(^{150}\). An example of Michael addition labelling is shown in Scheme 5.

Scheme 5: PMP 12 labelling of carbohydrates by Michael addition. The \(R_1\) group represents the rest of the glycans.

Saba et al (1999) investigated the use of PMP 12 labelling of sialylated glycans A1 and A2. In the analysis of the monosialoglycan A1, a 100-fold increase in sensitivity was observed for the PMP 12 labelled species over the native glycan with MS detection \(^{151}\). Although this labelling method results in an increase in sensitivity over native glycans, limitations of this labelling method are present due to the addition of two labels per glycan. This results in the separation in high performance
liquid chromatography (HPLC) methods being dominated by the label rather than the glycan, which is disadvantageous in separating isomers.\textsuperscript{143}

1.3.1.4 Commonly used labels

A variety of labelling, detection and separation methods may be used in the analysis of glycans. The differing methods of purification and detection that may be used rely on different moieties. Labels display analytical niches where they possess moieties that benefit analysis for one or a few methods, but these moieties will also be to the detriment of other methods. For this reason, a wide variety of labels are employed depending on the analysis being undertaken. \textbf{Table 2} lists some of the commonly used glycan labels.

\begin{table}[h]
\centering
\caption{List of chemical labels commonly used in glycan analysis. The group that takes part in the labelling reaction is circled.}
\begin{tabular}{|c|c|c|}
\hline
Fluorescent glycan labels & Structure & Information \\
\hline
\textit{Reductive amination} & & \\
2-AB \textsuperscript{10} & \includegraphics[width=1in]{2-AB.png} & This molecule is commonly used as a fluorophore in HPLC. It is used in MS but as it is less hydrophobic 2-AB \textbf{10} results in lower signals compared to other labels. 2-AB \textbf{10} labelling is routinely employed in glycan analysis and is commercially available in a kit form.\textsuperscript{152} \\
\hline
\end{tabular}
\end{table}
<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-anthranilic acid (2-AA) 13</td>
<td><img src="image1" alt="Structure" /></td>
<td>2-AA 13 carries a negative charge and is suitable for analysis in negative-mode MS but is also used in positive-mode in the detection of neutral glycans. This label is used in HPLC and capillary and gel electrophoresis analyses.</td>
</tr>
<tr>
<td>2-aminopyridine (2-PA) 14</td>
<td><img src="image2" alt="Structure" /></td>
<td>2-PA 14 is widely used in HPLC glycan detection for HPLC-MS. This label requires crystallization before derivatisation meaning this method takes longer than others.</td>
</tr>
<tr>
<td>2-aminoacridone (AMAC) 15</td>
<td><img src="image3" alt="Structure" /></td>
<td>AMAC 15 has a highly fluorescent aromatic region, which makes detection with FLD in picomolar ranges possible. This label ionises readily making it compatible with electrospray ionisation (ESI) MS and matrix assisted laser.</td>
</tr>
<tr>
<td><strong>3-aminoquinoline (3-AQ) 16</strong></td>
<td><img src="image1" alt="Chemical Structure" /></td>
<td>3-AQ 16 is used as a matrix in carbohydrate analysis by MALDI-MS. 3-AQ 16 is also rarely used as a N-linked glycans label, although has lower fluorescent efficiency than 2-AB 10.</td>
</tr>
<tr>
<td><strong>6-aminoquinoline (6-AQ) 17</strong></td>
<td><img src="image2" alt="Chemical Structure" /></td>
<td>This label was found to have similar properties to 3-AQ 16 for analysis of carbohydrates by HPLC.</td>
</tr>
<tr>
<td><strong>8-aminopyrene-1,3,6-trisulfonic Acid (APTS) 18</strong></td>
<td><img src="image3" alt="Chemical Structure" /></td>
<td>APTS 18 has three negatively charged sulphate groups, which makes it ideal for electrophoretic methods of separation. However, these charges prevent methods such as MALDI-MS.</td>
</tr>
<tr>
<td><strong>8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) 19</strong></td>
<td><img src="image4" alt="Chemical Structure" /></td>
<td>ANTS 19 is used in capillary electrophoresis (CE), as it provides pH</td>
</tr>
</tbody>
</table>
independent anionic charge. The presence of the sulphate groups results in poor ionisation in MALDI-TOF MS\(^\text{161}\).

<p>| 3-(acetylamine)-6-aminoacridine (AA-Ac) 20 | <img src="image" alt="Structure" /> | This label is suitable for MALDI-TOF MS analysis and CE. This label is also appropriate for FLD detection due to the intense fluorescence allowing detection in picomolar amounts(^\text{162}). |
|Procainamide (ProA) 21 | <img src="image" alt="Structure" /> | This is a commonly used label and is available in kit form. The tertiary amine group is an efficient charge forming group which means this label performs well in positive-mode MS by ESI or MALDI ionisation and is more sensitive in MS and FLD than 2-AB 10(^\text{163}). |
|Methyl-4-aminobenzoate (ABME) 22 | <img src="image" alt="Structure" /> | Not commonly used as a glycan label. Separation of ABME 22 |</p>
<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Structure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl-4-amino-benzoate (ABEE)</td>
<td><img src="image" alt="Ethyl-4-amino-benzoate" /></td>
<td>Another less commonly used label. This label showed increased sensitivity by a factor of 2 compared to native glycans and improved resolution and longer retention time compared to ABME 22.</td>
</tr>
<tr>
<td>Butyl-4-amino-benzoate (ABBE)</td>
<td><img src="image" alt="Butyl-4-amino-benzoate" /></td>
<td>The increased chain length results in longer elution times and a slight increase in sensitivity in negative mode MS compared to ABME 22.</td>
</tr>
<tr>
<td>5-Amino-2-naphthalenesulfonic acid (ANSA)</td>
<td><img src="image" alt="5-Amino-2-naphthalenesulfonic acid" /></td>
<td>The single negative charge facilitates the analysis of labelled oligosaccharides by negative mode MALDI-TOF. This label has superior resolution to APTS 18 in both capillary electrophoresis (CE) and HPLC.</td>
</tr>
</tbody>
</table>
**Carbamate rapid tagging**

| RapiFluor-MS (RF-MS) 26 | **N**-hydroxysuccinimide (NHS) carbamate facilitates faster labelling of glycan. RF-MS is suitable for analysis by HPLC with FLD and MS. |

**Quinoxaline formation**

| 1,2-diamino-4,5-methylene-dioxypicolone (DMB) 27 | This label is used solely for the analysis of sialic acid in HPLC-FLD, ESI and MALDI-TOF. |

**Michael addition**

| PMP 12 | Labelling is performed under alkaline conditions by Michael addition. This molecule can be used in both normal and reverse phase HPLC, and is suitable for LC-ESI-MS analysis. |

| 1-(2-naphthyl)-3-methyl-5-pyrazolone (NMP) 28 | Behaves similarly to PMP 12. This label shows increased sensitivity by |
ultraviolet (UV) detection and a faster reaction time compared with PMP 12.

**Hydrazine labelling**

<table>
<thead>
<tr>
<th>Phenylhydrazine 11</th>
<th>Phenylhydrazine 11 labelled glycans were analysed by MALDI and ESI in picomolar concentrations. This label gave quantitative labelling of glycans.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dansyl hydrazine 29</td>
<td>This label is commonly used for mono- and disaccharide analysis with CE- light induced fluorescence (LIF). Capable of detecting samples at 10 picomolar ranges.</td>
</tr>
<tr>
<td>Fluorenylmethoxy-carbonyl hydrazine 30</td>
<td>This label has a short reaction time, taking about 3.5 hours compared to 1-2 days that is typical of other hydrazine labelling. This label can used for</td>
</tr>
</tbody>
</table>
1.3.1.3 Purification of labelled glycans

As glycan labelling reactions are generally performed in an excess of labelling reagent to ensure high derivatisation efficiency it means that large concentrations of labelling reagents remain in the sample. Should these reagents remain in the sample during analysis it may produce signals during detection that complicate characterization. For this reason, following the modification of the glycan, the derivatisation reagent may be removed. The purifying of the labelled glycans forms a routine part of some glycomic analytical workflows. Various methods of removing these excess reagents are performed with some of the common methods mentioned in the following sections.

1.3.1.3.1 Precipitation purification of glycans

The first method of purification of a glycan is via acetone precipitation. This method arises from the solubility of the label in acetone but not of the labelled glycan. Precipitation is performed with the addition of water free acetone followed by centrifugation and removal of the acetone supernatant. This removes any acetone soluble impurities leaving pelleted oligosaccharides which can be extracted in ice-cold aqueous MeOH. This method has been applied in the purification of glycan labelled with 2-AB, 2-AA, AA-Ac, ABEE, ABBE, and ProA. This method may also be applied to the removal of proteins during the release of glycans.
1.3.1.3.2 Liquid-liquid extraction purification of glycans

Liquid-liquid extraction (LLE) is a method of purifying derivatized glycans by exploiting the relative solubilities of an analyte. In this example the purification is produced from the relative solubilities of molecule between two immiscible solvents such as an organic and aqueous solvent. In this method the derivatised glycans occupy the organic phase whereas contaminants soluble in the aqueous phase are removed. As glycans are polar, this method has particular application for glycans derivatized by methods that largely increase the hydrophobicity of the glycan such as permethylation, hydrazide labelling and Michael addition.

Desantos-Garcia et al (2011) purified permethylated glycans with LLE by first partitioning the samples between 500 mM sodium chloride (aq) and chloroform. The aqueous layer of this mixture was discarded before, water was then added and the aqueous layer was again discarded. This addition and removal of water was repeated and the remaining organic solution was dried in vacuo to yield purified permethylated glycan.

1.3.1.3.3 Size exclusion chromatography separation of glycans

Glycan purification may also be performed with size exclusion chromatography (SEC). This method uses a column packed with small porous beads. SEC is divided into two basic type, the first is gel permeation chromatography which uses a hydrophobic packed column and a non-aqueous mobile phase and the second is gel filtration chromatography which uses a hydrophilic packing material and an aqueous mobile phase. In the case of glycan purification, gel filtration chromatography is used with hydrophilic resins such as Sephadex or Sepharose.
In SEC, separation is a result of the differences in hydrodynamic volume of the analyte, which is the volume of a molecule when it is in solution \(^{180}\). The hydrodynamic volume of a molecules depends on how well the molecule interacts with the solvent and the molecular weight. Molecules with smaller hydrodynamic volumes diffuse further into the pores of the microporous resin are able to diffuse more into the pores of the bead as there is less steric hindrance \(^{181}\). Molecules with larger hydrodynamic volumes cannot access the pores and are confined to the outside of the resin. As the small beads enter the pores it results in increased retention on the column and smaller analytes elute later. In contrast as larger analytes do not enter the beads they elute faster \(^{181}\). Figure 22 illustrates the mechanism of separation for SEC. Gel filtration does encounter issues due to how oligomers are separated. As analytes are separated by hydrodynamic volumes, different species with similar hydrodynamic volumes elute at the same time. However this is also encountered in other methods of purification \(^{182}\).

![Figure 22: Representation of the mechanism of separation for SEC.](image)

SEC is particularly effective in the separation of large polysaccharides such as a starches, dextrans and GAGs \(^{183}\). Vanderschaeghe et al (2010) purified serum N-linked glycans post APTS 18 labelling using Sephadex G10 filled microplates or Sephadex G10 microtips with a mobile phase of DNA-500 Separation Buffer from
Schimadzu \textsuperscript{184}. Higel \textit{et al} (2013) purified 2-AA \textbf{13} labelled antibody glycans by gel filtration chromatography with Sephadex G-10 columns with a mobile phase of water \textsuperscript{185}.

SEC can also be employed in the purification of GAGs \textsuperscript{186}. The purification of heparin oligosaccharides was performed by Wang \textit{et al} (2012) with a TSK-GelG3000PWx1 column with 2M NaNO\textsubscript{3} as the mobile phase. This research was successful in creating a standard curve that could be used to calculate relative molecular mass properties of the polysaccharide in the microgram scale.

1.3.1.3.4 Paper chromatography purification of glycans

Paper chromatography is a method of purification that uses chromatography paper as a stationary phase for separation. Paper is made of cellulose, a polymer of glucose with the polymer chains presenting -OH groups. These -OH groups are associated with a thin layer of water bound to the surface. Paper chromatography is a form of partition chromatography between the mobile phase and the thin layer of water. Non-polar analytes will have lower attraction to the polar stationary phase and will travel further on the paper. Polar analytes will have higher attraction to the thin water layer and will travel less along the paper. A representation of paper chromatography is shown in \textbf{Figure 23}. 
Figure 23: A representation of ascending paper chromatography (left) and descending chromatography (right). The arrow denotes the direction of solvent movement.

Ascending paper chromatography can be performed in acetonitrile (MeCN) to remove any excess label from a labelling reaction. Following the ascending paper chromatography, the paper can be dried and washed with water to elute samples from the origin. Royle et al. (2002) used paper chromatography in the purification of O-linked glycans released by hydrazinolysis from bovine serum fetuin. They also used paper chromatography to remove excess 2-AB label after fluorescent labelling. This method was time consuming (typically 48 h) and suffers from limitations. Paper chromatography may not remove salts from the sample which hinders subsequent detection as salt is known to quench signals in some detection methods.

Descending paper chromatography is performed with prewashed filter paper to separate carbohydrates from impurities such as peptides and labelling reagents. Mobile phases used in the purification of carbohydrates in this manner are normally composed of mixtures of butanol: pyridine: water. After the initial separation, the sections of paper close to the origin are washed with water to collect glycans.
1.3.1.3.5 Solid phase extraction purification of glycans

The last method of glycan purification and a method routinely used for the purification is solid phase extraction (SPE) \(^{143}\). SPE is a method of separation that relies on the different affinities of analyte and interferents in solution to a sorbent solid phase. Several sorbents are used effectively for the purification of derivatized carbohydrates including amino, diol, sialo, cyano, hydrophilic interaction liquid chromatography (HILIC), c18 and PGC \(^{190}\). This process of purification by SPE is shown in Figure 24.

![Figure 24: Representation of glycan purification with SPE. Glycans from glycoproteins and released and derivatised. Derivatised glycans are then loaded onto SPE, the fluorescent label present in excess shown as the orange stars are washed away and glycans are eluted for further analysis.](image)

Glycans are released and labelled with fluorescent label which are represented by the orange shapes. The labelling reaction is then added onto the SPE sorbent and washing steps remove excess labelling reagents while the pool of derivatised glycans are retained. The pure pool of glycans can then be collected.
The variety of SPE sorbents retain analytes through different interactions. HILIC SPE retains molecules based on their hydrophilicity. Hydrophilic molecules such as glycans are retained whereas hydrophobic reagents are eluted in the washing steps. Glycans are loaded in organic solvents with small amounts of water and washing steps are performed with organic solvents with small amounts of water, for example MeCN and 20% water. Glycans are then eluted with polar solvents such as water.

Porous graphitized carbon (PGC) sorbents are another possible SPE sorbent used for purifying glycans post labelling, however this sorbent is most widely applied for the purification of underivatized glycans after release from the glycoprotein. The mechanism behind the purification by PGC is not well understood but has been optimized to be highly specific for glycans. PGC is not used as often due to a higher cost and the fact that, depending on the label used in derivatisation, removal of the excess reagent is not always possible.

Reversed phase (RP) sorbents are also used. RP-SPE retains molecules based on their hydrophobicity and so are especially applicable for methods that cause hydrophobic alterations such as permethylation or hydrazide labelling with large hydrophobic labels such as biotinamidohexanoic acid hydrazide (BACH). It is also commonly used for glycans released from cells and tissues to remove sodium dodecyl sulphate (SDS).

1.3.1.4 Separation of glycans

Following the purification of the glycans, they may be analysed, in some case prior to detection, the separation of the pool of analytes may be performed. This may be done to simplify analysis for example by making it possible to differentiate between
structural isomers. Many methods of glycan separation may be used during analysis depending on the desired detection methods as the conditions required by some separation methods prevents the use of some detection methods. The methods used may also be determined by the label that has been used for the derivatisation.

1.3.1.4.1 Separation of glycans by HPLC

HPLC is routinely employed for the separation of labelled carbohydrates and may be coupled with inline detection methods. HPLC is a varied discipline and many modes of HPLC exist. This is also true in the use of HPLC in the analysis of glycans where different modes of HPLC can be used. The methods of HPLC used in the analysis of carbohydrates are detailed in the following sections.

Normal phase HPLC (NP-HPLC) was the first method of HPLC used for analysis of glycans. This mode of HPLC uses a polar stationary phase such as silica, cyano and amino groups and a non-polar mobile phase. Interaction between the polar analytes and the polar stationary phase results in retention. In the context of glycan analysis, it means larger and more hydrophilic glycans display greater interaction with the polar stationary phase. This results in an elution order where glycans elute in the order of increasing size.

Another method of HPLC widely employed in glycan analysis is HILIC. HILIC is considered to share similarities with NP-HPLC as they both use polar stationary phases and shows the same elution order as NP-HPLC. In the case of HILIC, stationary phases are similar to NP: commonly diol, amino, cyano, amide or carbamoyl phosphate groups. HILIC sees differences to NP-HPLC as it uses polar mobile phases normally composed of aqueous mixture of organic salts and aprotic organic solvents.
PGC stationary phases may also be used in HPLC glycan analysis. PGC stationary phases were developed to overcome issues associated with silica based stationary phases, such as the instability of the bonded ligands. PGC is more inert compared to silica-based sorbents, and shows function over an wide range of pHs from 10 M acid to 10 M alkali and at high temperatures (>200 °C). In PGC retention is predominantly mediated by a combination of ionic, absorption and hydrophobic interactions. Mobile phases for PGC are mostly comprised of gradients of organic solvents with small volatile salts. PGC has been used in the separation of large, small, native and derivatised glycans. PGC is used in the separation of O-linked glycans released by β-elimination as these glycans do not have intact reducing ends for labelling. Labels contribute greatly to retention as glycans labelled with 2-PA when analysed with PGC produced a well resolved trace of separated labelled glycans allowing easy determination between structural isomers.

RP-HPLC is a widely used analytical technique in many laboratories. In RP chromatography, analytes are retained depending on their hydrophobicity with more hydrophobic molecules retained more and eluting later. As hydrophobicity is required for separation in RP it means traditionally RP has had less adoption in the field of glycan analysis as native glycans are very polar. Glycans have no interaction with the stationary phase and are eluted in the void. When glycans are analysed by RP, separation relies on the derivatised component of the glycan, therefore derivatisation is essential to provide sufficient retention. RP therefore has greater compatibility with derivatisation methods that produce the most hydrophobic products such as Michael addition and permethylation. Alternatively, ion pairing RPLC may be performed for the analysis of underivatized glycan as the mechanism of retention in this case is not reliant on the hydrophobicity of the glycan label. Ion pairing chromatography utilises an ionic pair reagent, a molecule which has both an ionic and a non-polar end. The non-polar side is held tightly to the stationary phase leaving the charged polar group exposed to the mobile phase. In this mode of HPLC, retention arises from the interactions
between the glycan and the ion pairing reagent tightly held to the stationary phase allowing the separation of glycan in RP without prior derivatisation.

1.3.4.1.2 Separation of glycans by anion exchange chromatography

Anion exchange chromatography separates glycans according to their charge $^{143}$. Anion-exchange chromatography of $N$-linked glycan is performed at neutral to slightly alkaline pH as under these conditions any carboxylic acids, sulfates and phosphates on glycans are charged $^{143}$. Anion exchange chromatography of GAGs can be performed at an acidic pH to decharge carboxylic acid groups meaning separation of GAGs is based on sulfation $^{198}$.

Weak anion exchange (WAX) chromatography separates species on relative binding affinities to a weak anionic matrix. $N$-linked glycans naturally exhibit negative charges, these arise from sialic acids, sulphates, phosphates and uronic acids while sulfate and uronic acid groups of GAGs produce a negative charge. Glycans are separated depending on the negative charge of the glycan with retention increasing with charge $^{199}$. This method gives differing separation between multiply sialylated glycans, mono-, di- and tri- sialylated glycans which elute at different times due to the increasing negative charge $^{199}$. Separated charged glycans can then be collected and separated further with NP liquid chromatography $^{143}$. Strong anion exchange chromatography can also be used for the separation of GAGs. In this application the charged analytes are separated due to ionic interactions to the charged stationary phase and the counterions in the mobile phase with retention based on the GAG being displaced by a competing salt which is often sodium chloride. GAGs with lower charge and a smaller size will elute first $^{200}$.

Glycan nanoprofiling is another possible application of WAX. Glycan nanoprofiling is a simultaneous quantification and characterisation using nanoLC-MS $^{201}$. Kalay et al
(2013) performed the nanoprofiling of AMC labelled fetuin glycans. This workflow employed a first separation step using preparative scale WAX chromatography to separate the glycans according to their sialic acid content resulting in five fractions for the non-sialylated, monosialylated, disialylated, trisialylated and tetrasialylated glycans. This was followed by NP-nanoLC-MS of each glycan pool. Kalay et al (2013) concluded that glycan nanoprofiling allowed rapid and detailed characterisation of N-glycomes.

High pH anion exchange chromatography (HPAEC) is version of anion exchange chromatography performed at more alkaline pHs, approximately pH 13. HPAEC uses quaternary ammonium bonded pellicular anion-exchange columns and may be used in the analysis of monosaccharides and glycans. Retention in HPAEC is affected by the number of charges on the analyte and the isomerism and polymerization of the glycan. Many carbohydrates are weak acids with pKaś in the 12-14 range, as HPAEC is performed at pH 13 there is a partial deprotonation of the hydroxyl groups resulting in oxyanions. Underivatized glycans with a free reducing end are retained more, due to the presence of the anomeric hydroxyl group, making it possible to separate underivatized and derivatised glycans.

HPAEC analysis of monosaccharides is performed isocratically with 10-20 mM aqueous solutions of sodium hydroxide. The analysis of glycans requires a stronger eluent than sodium hydroxide, in these cases, gradients of aqueous sodium acetate solutions are employed. Gradient methods of HPAEC are performed with a gradient of sodium acetate whilst keeping a constant concentration of sodium hydroxide.

Combination methods of HILIC and anion exchange has also been developed in the analysis of glycans. This combination technique is performed with amine bonded silica stationary phases or a strong anion exchange HPLC column with quaternary...
ammonium groups. The acidic pH of the mobile phase causes the bonded amine to partially protonate where it is able to cause separation by the anion exchange mechanism. Balancing between HILIC mode and anion exchange mode contributes to ionic interactions between both the tag and glycan which can be modulated by altering the ionic strengths of the eluents.

1.3.1.4.3 Separation of glycans by electrophoresis

The analysis of glycans can also be performed using electrophoretic methods such as CE which separates species based on their electrophoretic mobility under an applied voltage. The rate of mobility is proportional to the applied electric field, the charge, viscosity and size of the analyte. Greater field strengths cause greater mobility; smaller molecules with will migrate further than large molecules with the same ionic charge. For molecules of comparable size, but differing charges, the species with the greater charge migrate further. In the context of glycan analysis, mobility is a result of negative charges originating from sialic acid and groups such as sulphate. CE has some advantages over other methods of separation such as HPLC as it is a comparatively simpler instrument. HPLC has a parabolic flow while CE has a flat flow which results in superior peak resolution compared to HPLC due to the reduced band spreading and peak widths. A representation of this is shown in Figure 25.

![Figure 25: Representation of parabolic flow (left) and flat flow (right).](image)

Electrophoresis may also be performed in a gel. Gel electrophoresis is a versatile method for separating any saccharide that is negatively charged. This method
separates glycans due to their size, composition and glycosidic linkages \cite{206}. Gel electrophoresis has advantages over other methods as it is inexpensive, easy to perform, requires no prior purification and can be used with long oligomers \cite{206}.

1.3.1.5 Detection of glycans

Following the derivatisation and possible separation of glycans, they can be detected to characterize and determine the components of the samples. Some detection techniques provide detailed information on the analyte, allowing complete structural determination, whilst others, themselves not providing structural data, give information that structure can be inferred from by comparing to other data. A variety of detection methods exist and may be used depending on the methods of derivatization or the method of separation preceding the detection.

1.3.1.5.1 Optical detection of glycans

The addition of a chromophore or fluorophore is common during derivatisation as they are a common feature of glycan labels. The addition of the fluorophore means it is possible to detect these samples by optical methods. Ultraviolet visible (UV-Vis) detection and FLD detection are commonly performed in-line with HPLC. UV-Vis can be performed with a photodiode array detector or a variable wavelength detector. Light is emitted by a lamp and passed through a monochromator at a set wavelength. The sample is pumped through a flow cell and irradiated. The absorption of light is detected by the photodetector. Absorption of light indicates the presence of the analyte. The degree of response is proportional to the concentration of the analyte. Limitations with UV-Vis detection include nonspecificity as many molecules absorb light in similar ranges. For this reason, UV-Vis detection is normally used in conjunction with other methods of detection such as MS \cite{143}. 

55
FLD is the most common optical detection method and may be used to improve specificity and sensitivity in glycan analysis\(^{143}\). Whereas UV-Vis detects the absorption of light, the detector response in FLD is caused by detection of emitted light. This is more specific than UV-Vis as it requires the analyte to meet two requirements to be detected, the analyte needs to match the specific excitation \((\lambda_{\text{Ex}})\) and emission \((\lambda_{\text{Em}})\) wavelengths\(^{207}\).

The lamp emits light that is passed through an excitation monochromator, this single wavelength of light excites the eluent being pumped through the flow cell\(^{208}\). The excited sample emits a photon at a longer wavelength and passes through an emission monochromator before it is detected (Figure 26). FLD shows sensitivity 10 - 1000 times higher than what is seen with UV.

Figure 26: Diagram representing FLD\(^{208}\).
FLD can be performed with a laser as the light source. Laser induced fluorescence (LIF) is similar to FLD mentioned above, but differs as the lamp is replaced as the excitation sources with a laser. This method is commonly used in conjunction with CE as LIF requires only small amounts of sample due to the high sensitivity of the detection method. 209. This method has been used very successfully in the quantitative and qualitative analysis of saccharides 210.

1.3.1.5.2 Pulsed amperometric detection of glycans

Glycans may also be detected by pulsed amperometric detection (PAD). Amperometry is a electrochemical detection technique based on the measurement of a current caused by the oxidation and reduction of an electroactive species when subjected to an electric field. In single-potential amperometry a current is pass between two electrodes in the column eluent, as an analyte passes between the electrodes the current changes as the analyte is oxidised at the anode or reduced at the cathode. PAD is a form of amperometry used instead of single-potential amperometry to prevent fouling of the electrode which can occur with single-potential amperometry which reduces sensitivity 211. PAD detection is performed with three cell electrodes. The three electrodes are the working electrode, the reference electrode and the auxiliary electrode 212. The reference potential provides the ability to measure the working potential without passing current through itself while the auxiliary electrode provides a path for current to flow in the electrochemical cell 213. Detection in PAD is performed by a applying a working potential to the working electrode for a short time. Oxidation of the analyte results in a change in the output current with the change being proportional to analyte concentration 214. The working potential is then followed by a higher or lower potential which is performed to clean the electrode 211.

This method is used almost exclusively alongside HPAEC as the analysis of oligosaccharides via PAD requires a high pH (>pH 12) for carbohydrates to become
charged, this makes it ideal in combination with HPAEC \textsuperscript{202}. HPAEC is also incompatible with other detection methods as the high salt environments required in HPAEC prevents direct coupling with detection methods such as MS without prior desalting \textsuperscript{215}. PAD has been observed to result in well resolved analysis, Kotani and Takasaki (1998) investigated the analysis of 2-AB \textbf{10} labelled asialooligosaccharides by HPAEC with PAD and FLD. They reported that analysis by HPAEC-PAD had superior resolution than samples detected by FLD \textsuperscript{216}.

1.3.1.5.3 Mass Spectrometric detection of glycans

The analysis of derivatized glycans is routinely performed by MS. MS is a method of analysis that detects the mass to charge ratio of an ion allowing determination of the exact molecular weight of a compound \textsuperscript{217}. The ionisation in MS may be performed by a variety of methods but common methods of ionisation in glycan analysis are ESI or MALDI as these are soft methods of ionisation \textsuperscript{218}.

In ESI, species are ionised in the gaseous phase by protonation or cationization. A continuous stream of sample is passed through a capillary tube at high voltage in the ESI source producing a mist of highly charged droplets \textsuperscript{219}. Charged droplets pass down a pressure gradient where the ions are accelerated into the mass analyser and are detected \textsuperscript{219}. ESI may also be used to perform MS/MS or MS\textsuperscript{2} to give further information on structural isomers and branches. In MS/MS, parent ions are fragmented in a collision cell, these fragments are detected at either a second mass analyser or by a tandem mass spectrometer \textsuperscript{219}.

In MALDI, ionisation relies on the principle of laser desorption ionisation. This is the formation of ions by irradiation with a high intensity laser. The addition of a matrix, a small molecule that has a strong absorbance at the wavelength of the laser, developed this irradiation process into MALDI \textsuperscript{220}. The matrix is mixed with the
analyte on a metal target plate, once dried, the spot of the mixture is irradiated by a pulsed laser beam producing a substantial burst of ions with each laser pulse which can then be detected. The ionised analyte is then accelerated and focused and mass of the analyte is determined in the flight tube. Detection in MALDI is widely performed with time of flight (TOF). Lighter mass ions travel faster in the flight tube compared to heavier ions, the difference in the time taken to traverse the flight tube can be used to determine the mass. A representation behind the ionisation and detection in MALDI is shown in Figure 27.

![Diagram representing MALDI-TOF detection.](image)

Figure 27: Diagram representing MALDI-TOF detection.

Another method of mass determination in MS uses a quadrupole. A quadrupole is a mass analyser composed on four parallel rods positioned equidistant from a centre axis, the rods are charged with opposite rods carrying like charges. The oscillating electrical field in the rods are changed to stabilize or destabilize ions. Ions travel
through the quadrupole in a corkscrew path. Only ions with selected m/z value successfully achieve a stable trajectory through the central axis to reach the detector. Ions at other m/z values have unstable trajectories through the centre axis and collide with the rods or are expelled from the quadrupole. The specific ion that can successfully travel through the quadrupole can be changed depending on the voltage applied to the rods 221.

1.4 Aims of the thesis

The methods of glycan analysis mentioned in this chapter form the workflows commonly used for the analysis of N-linked glycans. Research in this thesis focuses on the analysis of N-linked glycans by creating tools to develop the N-linked glycans analytical workflow. This was performed by synthesising new multifunctional glycan labels that retain the amine functional group required for labelling but also feature an alkyne group allowing further analysis of the labelled glycan to be performed post analysis. These HILIC and RP analysis of glucose homopolymer (GHP), RNase B glycans and IgG glycans labelled with commercially available labels and multifunctional alkyne derivatives were compared to investigate the effectiveness of these novel multifunctional glycan labels. The ability to conjugate the additional alkyne group on the glycan labels with an azide was also investigated to determine whether it is possible to introduce further analysis of collected labelled glycans.

Finally, this thesis investigates the use of commercially available ortho-phenylenediamine (OPD) derivatives for quinoxaline derivatisation in the analysis of free sialic acid (FSA). RP analysis of the FSA labelled with commercially available OPD derivatives and a commonly used FSA label was performed to determine which label gave the most sensitive and stable product in RP-HPLC analysis.
The research in this thesis is summarised in Figure 28 which presents the experiments, methods of analysis, glycans and labels that are used.
Figure 28: A flowchart summarising the research aimed to be undertaken in this thesis.
Chapter 2 - Synthesis of multifunctional fluorescent labels for glycan analysis

2.1 Background

The workflows detailed in the previous chapter are well-established methods for the characterization of glycans in a research and industrial context. However, aspects of the current workflows such as the label used and the efficiency of the labelling reaction can cause issues of poor detection in analyses with FLD and MS. Labels often display “analytical niches” whereby they exhibit characteristics that benefit analysis by a few specific methods but perform less well in others meaning commercially available glycan labels can exhibit differences in sensitivity depending on the method used.143, 222-224.

Pabst et al (2009) labelled a biantennary non-sialylated A\(^4\)A\(^4\) glycan with 2-AA 13, APTS 18 and ProA 21 to investigate the detection of the labelled glycan after SPE purification. When analysed with positive mode ESI, the (M+Na)\(^+\) signal of the ProA 21 labelled glycan was two-fold higher than the 2-AA 13 labelled glycans, with the APTS 18 labelled glycan not detected. ProA 21 labelled analytes in positive mode MALDI had signals corresponding to the (M+Na)\(^+\) 150% higher than 2-AA 13, while no signal for the APTS 18 labelled glycan could be detected.176

Harvey (2000) compared the signals of glycans labelled with ABBE 24, AMAC 15 and ProA 21 labelled GlcNAc\(_2\)Man\(_5\) by both MALDI and ESI. In this research, the AMAC 15 labelled samples showed the highest signal in MALDI, 150% higher than the ABBE 24 labelled samples and two-fold higher than the ProA 21 labelled species. In ESI, the ABBE 24 labelled species showed the highest detector signals, four-fold higher and twelve-fold higher than the AMAC 15 and ProA 21 labelled species respectively.225
With FLD analysis, the research by Pabst et al. (2009) saw ProA 21 labelled species having a two-fold increase in signal compared to 2-AA 13 when analysed by HPLC-FLD, and a 150-fold increased response compared to APTS 18. Dhume and Anumula (1998) investigated the difference in FLD of fluorescent labels 2-AA 13, AMAC 15 and 6-AQ 17 and found 2-AA 13 was four-fold more intense than AMAC 18 and three-fold compared to 6-AQ 17.

In the research by Pabst et al. (2009) the \([\text{M-H}]^3\) signal was seven-fold higher for 2-AA 13 labelled glycans compared to ProA 21 labelled glycans and in negative mode ESI the 2-AA 13 labelled glycans had a four-fold higher detector response compared to the ProA 21 whereas the APTS 18 labelled samples as they are triply charged could not be detected.

The negative charges on 2-AA 13 and APTS 18 mean they can be used with PAGE and CE as these are method that benefit from negative charges. Fomin et al. (2020) investigated the use of fluorescent negatively charged label added by reductive amination for the analysis of maltodextrin oligosaccharides by electrophoresis as both 2-AA 13 and APTS 18 are fluorescent. However, 2-AA 13 is not fluorescent by argon laser whereas APTS 18 is. As CE is often performed with LIF or ESI-MS it means APTS 18 labelled samples are compatible with CE-LIF and will be detected while 2-AA 13 is not. Table 3 summarises the various analytical niches of the most commonly used glycan labels according to Ruhaak (2010) as well some selected other labels relevant to the research in this thesis.
Table 3: A table of commonly used labels and labels relevant to the research in this thesis and the analytical technique they are employed in \(^{143}\).

<table>
<thead>
<tr>
<th></th>
<th>HPLC</th>
<th>Electrophoresis</th>
<th>Anion exchange</th>
<th>FLD</th>
<th>LIF</th>
<th>+ ESI</th>
<th>- ESI</th>
<th>MALDI</th>
</tr>
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<tbody>
<tr>
<td>2-AB 10</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>2-AA 13</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>2-PA 14</td>
<td>✓</td>
<td></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<td></td>
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<tr>
<td>6-AQ 17</td>
<td>✓</td>
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<td>✓</td>
<td>✓</td>
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<td>✓</td>
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<tr>
<td>APTS 18</td>
<td>✓</td>
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<td>✓</td>
<td>✓</td>
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<tr>
<td>ANTS 19</td>
<td>✓</td>
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<td></td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>ProA 21</td>
<td>✓</td>
<td></td>
<td></td>
<td>✓</td>
<td>✓</td>
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<td></td>
<td></td>
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<tr>
<td>ABBE 24</td>
<td>✓</td>
<td></td>
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<td>✓</td>
<td>✓</td>
<td></td>
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\(^{143}\)
This demonstrates the differences encountered between the sensitivity of labels in different detections methods such as FLD and MS. These differences in some cases, will mean that detection methods are not sensitive enough to detect some glycans. The effort to detect these low abundance glycans has led to the production of new labels with greater sensitivity. RF-MS 26 is a glycan label that was developed to overcome low MS ionization and confidently detect glycans at a lower concentration. The large hydrophobic quinoline moiety in the label imparts high ionization efficiency.

2.2 Aims and discussion of the research problem

Existing glycan labels are ideal for the analysis of samples in high concentrations, but in cases where the concentration of glycans are low, it makes detection difficult. The aim of this chapter is to synthesise a multifunctional label that will improve the analysis of glycans by providing the ability for further reactions post analysis. A multifunctional label is defined as a label that can be used in glycan analysis workflows but contains a group that can be covalently reacted with another molecule to introduce further uses and functions. In this thesis, further uses were aimed at improving the detection of the low abundance glycans. This would be investigated by collecting samples post detection, these collected glycans can then be reacted with a group which will increase ionisation efficiency and the detector response for these glycans, and make detection at lower concentrations possible.

The work in this chapter details the design and synthesis of novel multifunctional glycan labels which bear an additional functional handle allowing further derivatisation following HPLC analysis. The creation of these multifunctional glycan labels had some requirements. The label must remain functional and reliable as a glycan label in current HPLC workflows and, in order to enable further downstream reactions, it would require an additional functional handle which allows for
chemoselective reactions at the newly installed functional group whilst causing no changes to the glycan. This group must also allow reactions in polar solvents such as water, as glycans are often poorly soluble in non-polar organic solvents. Three potential reactions were considered which fulfilled the criteria of being chemoselective and could be performed in water: the copper catalysed alkyne-azide cycloaddition (CuAAc) reaction, the strain promoted azide-alkyne cycloaddition (SPAAC) reactions and the tetrazine ligation.

2.2.1 Copper catalysed azide-alkyne cycloaddition reaction

The CuAAc is a reaction between an azide and a terminal alkyne, producing one of two potential triazole regioisomers depending on the conditions of the reaction. **Scheme 6** shows the products of this reaction. When proceeding with heat, a mixture of the 1,4- and 1,5-regioisomers of the triazole is formed. If the reaction is performed in the presence of a copper catalyst the reaction results in solely the 1,4-regioisomer.

![Scheme 6: CuAAc reaction products](image)

The copper free azide alkyne cycloaddition is an example of an azide-alkyne Huisgen cycloaddition reaction. This is a 1,3-dipolar cycloaddition reaction between a 1,3-
dipole and dipolarophile resulting in a five membered ring. The mechanism of the copper free azide reaction is shown in Scheme 7.

Scheme 7: The mechanism of the 1,3 dipolar cycloaddition between an alkyne and an azide forming the 1,4 and 1,5 triazole regioisomers.\(^{230}\)

The copper catalysed variety of this reaction in Scheme 8 relies on the formation of a copper alkyne bond with the Cu (I) catalyst. This copper alkyne bond reacts with the azide moiety to form a six membered ring before copper is lost and a triazole is formed.\(^{229}\)
The presence of Cu (I) is required for this reaction. It was originally performed with copper iodide (Cul), however Cul is air sensitive and iodine is known to react with alkynes and azides under certain conditions. The reaction with the alkyne group forms an unproductive polymeric copper acetylide complex which prevents the cycloaddition reaction. The most common form of Cu (I) species used now is produced in situ using CuSO$_4$•5H$_2$O and a reducing agent, typically sodium ascorbate in a water/alcohol mixture. The presence of the organic cosolvent ensures solubility of hydrophobic reactants.

2.2.2 Strain promoted azide-alkyne cycloaddition

The second reaction mentioned above, the SPAAC reaction, occurs between azide and an alkyne when the alkyne moiety forms part of a strained system with electron withdrawing groups. For example, when an alkyne forms part of a cyclooctyne ring with neighbouring benzene or fluorine groups such as bicyclonon-4-yne (BCN) and 3,3-difluorocyclooctyne (DFCO) in Figure 28.
Figure 29: Structures of as BCN 31 and DFCO 32.

The ring constrained system promotes the same triazole forming cycloaddition reaction in a concerted 3 + 2 cycloaddition reaction with the same mechanism as a Huisgen 1,3-dipolar cycloaddition. An example of the SPAAC reaction between BCN 31 and benzyl azide 33 is shown in Scheme 9. This reaction is well suited for use as a biorthogonal reaction due to the fact that metal ions are not required.

Scheme 9: The SPAAC mechanism and the representation of the SPAAC reaction between BCN 31 and benzyl azide 33.
2.2.3 Tetrazine ligation

The final reaction is the tetrazine ligation which is an example of an inverse electron-demand Diels-Alder reaction (IEDDA). This reaction is a [4+2] cycloaddition forming a six-membered ring between an electron rich dienophile and an electron poor diene, the inverse of the Diels Alder reaction which is performed with an electron rich diene and electron poor dienophile. The mechanism for this reaction is disputed, with both a stepwise and concerted mechanism proposed.

The 1,2,4,5-tetrazine was amongst the first diene applied in this reaction, this group reacts with a variety of strained dienophiles forming a dihydropyridazine product with the loss of nitrogen shown in Scheme 10\textsuperscript{239}. The reaction has large applications owing to the fast kinetics of the reaction at low concentrations and the ability to perform the reaction without metal\textsuperscript{239}. The frontier molecular orbital theory explains the reaction kinetics by the energy gap between the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO). A pair of reactants with a small energy difference between the HOMO dienophile and the LUMO of the diene results in a faster reaction.

The IEDDA reaction also has a large variation of possible dienophiles including cyclooctene, cyclooctyne, cyclopropene and norbornene moieties and dienes including tetrazines, triazine, pyridazines and pyridine\textsuperscript{241}. The product of the tetrazine ligation with cyclooctene and norbornene is shown in Scheme 11.
Scheme 11: Examples of tetrazine ligation reaction between tetrazine and a) cyclooctene and b) norbornene $^{242, 243}$.

2.2.4 Evaluation of the linking reactions reactivity

CuAAc, SPAAC and tetrazine ligation are powerful linking reactions, versatile for linking biocompatible natural molecules. They are regiospecific, modular, wide in scope, tolerant to many reaction conditions over a wide range of pHs, insensitive to water and oxygen, and generate only inoffensive by-products that can be removed via nonchromatographic methods $^{232, 244, 245}$. These reactions are robust, one-pot, facile, fast methods of linking molecules with a high thermodynamic driving mechanism which results in a high yield of product and a fast reaction. Both CuAAc and the tetrazine ligation are fast, with a reaction rate of $10^{-1}$–$100$ M$^{-1}$s$^{-1}$ for CuAAc and a reaction rate of $10^2$–$10^5$ M$^{-1}$s$^{-1}$ for the tetrazine ligation $^{246}$. The copper free SPAAC reaction suffers from slow reaction kinetics at low concentrations of biomolecules with reaction rates of $0.0012$–$0.96$ M$^{-1}$s$^{-1}$ $^{246}$. However, overall the CuAAc reaction was ultimately preferred due to the smaller addition to the label that would be required for CuAAc as this was expected to not change the chemical properties such as polarity and fluorophoricity, ensuring the label stays effective for the analysis of glycans.
2.2.5 Further potential uses of the linking reactions in glycan analysis

Other potential use for the multifunctional label may be to conjugate collected labelled glycan to proteins that have been azides incorporated onto the protein. This may allow selective addition of glycan structures to proteins in live cells for imaging purposes.

These reactions have become very useful in the context of imaging and monitoring glycosylation in the field of glycomics \(^{247}\). CuAAc has been used to monitor the dynamics of cell glycosylation. Cells are cultured in a media supplemented with the sialic acid precursor peracetylated \(N\)-(4-pentynonyl) mannosamine. Peracetylated \(N\)-(4-pentynonyl) mannosamine is taken up into the cell and is hydrolysed where it is then used in the cells’ biosynthetic pathway and incorporated into the glycan. This is shown in Figure 30. The glycans synthesised contain alkyne groups which are then labelled with a fluorogenic azide which allows them to be fluorescently imaged, the intensity of fluorescence can be used to calculated the glycosylation due to increased glycosylation resulting in increased fluorescence after conjugation of the alkyne group \(^{248}\).
2.3 Results and discussion

2.3.1 Structure of glycan label derivatives \textbf{35} and \textbf{36}

Two alkyne labels were designed, both derivatives of currently used labels for the reductive amination of glycans which undergo the CuAAC reaction.
Derivative 35 is based on ProA 21, a label commonly used for the analysis of glycans in industry due to the superior charge forming in ESI compared to labels such as 2-AB 10. In MS, labelling with ProA 21 typically gives detector signals thirty-fold higher than 2-AB 10 responses due to the diethylamine section of the molecule. With FLD detection, detector response signal is three-fold higher compared to 2-AB 10.

21 was modified by the addition of the terminal alkyne group at the aliphatic portion by replacing one of the ethyl groups with a propargyl moiety that would take part in the CuAAC reaction. This was the smallest change required for any of the three potential reactions, increasing mass of the label by only 10 Da. This modification was envisaged not to significantly alter the size or physicochemical properties of the label such as the partition coefficient and ionisation efficiency. These properties are crucial for the label to analyse glycans; the partition coefficient is a measure of solubility in polar and non-polar solvents. Solubility of the novel label in polar solvents is important as glycans are highly polar and poor dissolution of the label in water may prevent its use in glycan analysis. However, if the label is too soluble in polar solvents, it may result in poor ionisation in ESI due to the hydrophobic bias seen in MS. How readily a molecule ionises is another important physicochemical property in a glycan label. The more readily a label ionises, the greater signal is produced and the easier the detection of glycans at lower abundance glycans will be.

The fundamental groups in the ProA 21 structures are shown in Figure 32. The amine in the blue circle is required for covalently bonding onto the carbohydrate during labelling, the orange circle is the region responsible for the fluorophoric properties.
of the label, responsible for the detection by fluorescence. The yellow circle contains
the aliphatic region of the label, this hydrophobic group benefits the ionisation of the
label. The formation of a charge may also arise from the protonation of the tertiary
amine group. The new label retains the presence of these groups but also features
the alkyne group in the green circle capable of reacting by CuAAC.

![Diagram]

Figure 32: Example of the moieties in ProA 21 and its derivative 35. The amine in the blue
circle: covalently bonds onto the carbohydrate during labelling; orange circle: region
responsible for the fluorophoric properties of the label; yellow oval: aliphatic
hydrophobic region of the label benefits the ionisation in MS; green circle: undergoes
CuAAC reaction.

Compound 36, a derivative of p-nitrophenyl, has previously been synthesised in the
laboratory and tested as a potential glycan label. This label has a similar
structure to
ABBE 24\textsuperscript{165}. The presence of the alkyl chain benefits sensitivity in MS. Modification
of the aliphatic region follows the most logical procedure as modification of this
regions retains the groups necessary for the label to perform similarly to the parent
compound. The groups responsible for the same activity as mentioned previously are
shown in Figure 33.
2.3.2 Synthesis of ProA derivative 35

Two methods used in the synthesis of 21 from the literature are shown in Scheme 12 and Scheme 13. These methods of synthesis start with the formation of the amide bond forming nitroprocainamide 39 before the hydrogenation forms the final product.

Scheme 12: Synthesis for ProA 21 performed by Rasheed et al.\textsuperscript{249}

The synthesis of 21 in Scheme 12 was performed by Rasheed et al. (2015) and performs the amide formation with silica in solid phase with an 82% yield. The hydrogenation was performed in 95% yield by refluxing nitroprocainamide 39 with iron in ethanol.
Scheme 13: Synthesis of ProA 21 performed by Ramesh et al.\textsuperscript{250}

The synthesis of 21 in Scheme 13 developed by Ramesh et al (2015) is performed in neat N,N-diethylethylenediamine 38 with ammonium nitrate at 50 °C for 12 h with a yield of 98%. The hydrogenation was performed with palladium on carbon in MeOH with ammonium nitrate at 95% yield\textsuperscript{250}.

After considering the syntheses in Scheme 12 and Scheme 13, the retrosynthetic analyses of 35 was performed. Two possible synthetic routes are shown in Scheme 14 and Scheme 15.

Scheme 14: Retrosynthetic analysis of 35.

The key elements of this strategy were the formation of the amide bond, and the addition of the propargyl group. Scheme 14 shows one example of the
retrosynthesis, in this route the forward reaction starts with the addition of the propargyl group between prop-1-yne synthon and $N$-ethylethylenediamine synthon. The product of that reaction, $N^1$-ethyl-$N^1$-(prop-2-yn-1-yl)ethane-1,2-diamine is then used in the amide formation reaction with 4-aminobenzoic acid synthon yielding novel label 35 as product. An alternative retrosynthetic analysis of 35 via a second route is shown in Scheme 15

![Scheme 15: Retrosynthetic analysis of 35.](image)

The retrosynthetic analysis of 35 in the second route in Scheme 15 differs in the order of the reactions in the forward synthesis. This approach starts with the formation of the amide group in a reaction between 4-aminobenzoic acid synthon and $N$-ethylethylenediamine synthon followed by the addition of the propargyl group to yield novel label 35.
Preparation of multifunctional label 4-amino-N-(2-ethyl(prop-2-yn-1-yl)amino)ethyl)benzamide 35 is shown in Scheme 16 over 5 steps. The synthesis began with the tert-butyloxycarbonyl (BOC) protection of 41 producing the BOC-protected product 46 (85%). 46 was then activated with NHS to produce the activated ester BOC-4-aminobenzoic acid NHS ester 47 (87%). The amidation of 47 with N-ethylethlenediamine 44 produced BOC-desethylprocinamide 48 which was used crude in a successful reaction with propargyl bromide 49 to form the BOC-protected final product 50. The deprotection of 50 was performed with trifluoroacetic acid (TFA) in dichloromethane (DCM), this acidic mixture was neutralised with amberlite and the crude deprotected label was purified by column chromatography to yield 35 as an oily yellow solid (82%). The various reactions attempted that culminated in the development of the final synthesis are highlighted in the following sections.

2.3.3 Optimisation of amide formation
As detailed in the retrosynthetic analysis, the formation of the amide bond was a crucial part of this synthesis. The formation of the amide bond was first attempted via a method developed by Nazih et al (2002)\textsuperscript{251}. This reaction is a one-pot conversion of a BOC-protected amine and an acid chloride to form an amide product (Scheme 17).

![Scheme 17: Conditions for the one-pot conversion reaction of a BOC protected amine.](image)

The BOC protection of amine 44 was performed in Scheme 18 which would be used for the further reactions.

![Scheme 18: BOC-protection of N-ethylethylenediamine 44.](image)

The BOC protection in Scheme 18 was performed successfully in 82% yield with a method developed by Lee et al (2007) for selective amination of diamines\textsuperscript{252}. The product of this reaction was used in the BOC conversion reaction with the method developed by Nazih et al (2002) in Scheme 19.
82

Scheme 19: A one-pot conversion reaction of BOC protected molecules.

The applicability of the method developed by Nazih et al. (2002) in this synthesis was tested between a reaction of tert-butyl (2-(ethylamino)ethyl)carbamate 51 and 4-nitrobenzoyl chloride 52 however this reaction produced a mixture of inseparable products and was not pursued. Instead, an amide synthesis by Amaczyk and Fino (1996) was attempted. In this example, the amide was formed between N-ethylethylenediamine 44 and acid chloride, 4-nitrobenzoyl chloride 52, to synthesise nitrodesethylprocainamide 53 Scheme 20.

Scheme 20: Synthesis of nitrodesethylprocainamide 53.

The synthesis of 53 in Scheme 20 was performed with NHS and triethylamine (NEt₃) in THF. 52 was converted in situ to the NHS ester of 4-nitrobenzoic acid as an activated version of the acid. Amines are highly reactive with NHS esters and are used in amine crosslinking to create stable amide bonds. The use of this ester results in faster reaction times due to the presence of the better leaving group. The reaction of the NHS ester with N-ethylethylenediamine 44 produced product 53 which was recrystallized to produce pure product in 77% yield.

2.3.4 Addition of the propargyl group
Following the synthesis of nitrodesethylprocainamide 53, the hydrogenation of the nitro group was required to generate the tertiary amine responsible for labelling covalently onto the glycan. This hydrogenation could be performed before or after the addition of the alkyne moiety. The route with hydrogenation of the nitro group following the addition of the alkyne group is shown in Scheme 21.

Scheme 21: Representation of the problems with hydrogenation of the nitro group.

The second route performs the hydrogenation of the nitro prior to the addition of the propargyl group. This addition of the propargyl group would also cause addition of the alkyne group on the newly formed amine group. This means that protection of the amine group would be required to ensure selective addition of the propargyl group onto the secondary amine of 53. This route is shown in Scheme 22.
Scheme 22: Synthesis of BOC-protected final glycan label 35.

The route outlined in Scheme 22 requires additional protection of the amine group to prevent a reaction with the propargyl group at this newly created amine 45. As this route added unnecessary steps to the synthesis, an alternative to the nitro group was used.

Scheme 23: Synthesis of BOC-4-aminobenzoic acid NHS ester 47; Reagents and conditions: a) (BOC)$_2$O, NEt$_3$, H$_2$O/Dioxane, RT, 24 h (85%) b; NHS, DCC, THF, RT, 16 h (87%).

Instead the route in Scheme 23 was performed using 4-aminobenzoic acid 41 rather than 4-nitrobenzoyl chloride 52. This eliminated the need for hydrogenation later in the synthesis. The synthesis of the activated ester 47 started with the BOC-protection of 4-aminobenzoic acid in 85% yield. The addition of NHS was performed with DCC as the carbodiimide crosslinker with a yield of 87%. The use of DCC was beneficial as
the crosslinking reagent as the addition of the NHS group resulted in the formation of dicyclohexylurea which is insoluble in most organic solvents and could be easily removed by filtration \(^{256}\). The NHS activated ester 47 product was then used in the amidation with \(N\)-ethylethylenediamine 44 as shown in Scheme 24.

![Scheme 24: Synthesis of crude BOC-desethylprocainamide 45.](image)

Compound 48 was synthesised in high enough purity via this route to use in subsequent reactions without further purification.

2.3.4.1 Propargyl group addition in the synthesis of multifunctional label

The retrosynthetic analysis in Scheme 14 and Scheme 15 encountered uncertainty in whether the addition of the propargyl moiety should be performed pre or post formation of the amide. The synthetic route in Scheme 25 is the approach attempted for the addition of the propargyl moiety prior to the formation of the amide bond.

![Scheme 25: Synthesis of \(N\)-ethyl-\(N\)-(prop-2-yn-1-yl)ethylenediamine 56. Reagents and conditions: a) 1) HCl, \(H_2O\), MeOH RT, 0.5 h, 2) (BOC)\(_2\)O, MeOH, RT, 1.5 h (82%); b) NEt\(_3\), MeOH, RT, 48 h (40%).](image)
The addition of the propargyl group was first attempted via a dehalogenation substitution reaction with propargyl bromide shown in Scheme 26. The reaction performed with the BOC-protected amine 53 resulted in selective addition of the propargyl group solely on the secondary amine in 40% yield.

Scheme 26: Synthesis of BOC protected final product 45 with N¹-ethyl-N¹-(prop-2-yn-1-yl)ethane-1,2-diamine 54.

Addition of the propargyl group was followed by removal of the BOC-group with TFA to produce N¹-ethyl-N¹-(prop-2-yn-1-yl)ethane-1,2-diamine 57. This was used in further reactions with 47 (Scheme 26) performed with NEt₃ in THF at RT for 24 h. This synthesis has a low yield of 39% which may have been caused by the high pH present from the deprotection reaction. Due to the low yield the conditions outlined in Scheme 26 were not employed.

A second method of propargyl group addition was attempted via reductive amination of 51 with propargyl aldehyde and sodium cyanoborohydride in borate buffered saline (BBS) shown in Scheme 27.

Scheme 27: The proposed addition of a propargyl group by reductive amination with the in-situ generation of propargyl aldehyde 56.
Originally this oxidation of propargyl alcohol with Dess-Martin periodinane (DMP) was attempted separate from later reactions, but attempts to isolate the propargyl aldehyde product 56 was difficult due to the volatility of the product. Instead the in-situ generation and one-pot reaction of the propargyl aldehyde was attempted. The resulting reductive amination reaction in BBS resulted in no reaction due to issues thought to be with the solubility of BOC-N-ethylethylenediamine 51 in aqueous solution, so this method was not preferred.

2.3.4.2 Addition of the propargyl group post amide formation

The addition of the propargyl group post formation of the amide that was tested is shown in Scheme 28.

![Scheme 28: Addition of the propargyl group forming the BOC-protected final product 50.](image)

The reaction of BOC-desethylprocainamide 48 with propargyl bromide 49 and potassium carbonate in MeCN gave the BOC-protected final product in 82% yield. As this route provided higher yields than the previous methods attempted this method was selected for the label synthesis.

2.3.5 Synthesis of 4-aminobenzoic acid butyl ester 24 derivative

The synthesis of compound 36 was carried according to protocols previously established in the group (Brzezikca et al (unpublished)) (Scheme 29)
Scheme 29: Synthesis of novel label 36; Reagents and conditions: a) (BOC)_2O, NEt₃, H₂O/Dioxane, RT 24 h (85%); b) NHS, DCC, THF, RT, 16 h (87%); c) NEt₃, DCM, RT, 16 h (61%); d) TFA : DCM, RT, 3 h (quant)

Preparation of novel label 4-amino-N-(pent-4-yn-1-yl)benzamide 36 is shown in Scheme 29 over 4 steps. The synthesis began in the same manner as 35 with the BOC-protection of 41 (81%) then activation of the BOC-protected product 46 with NHS to produce the activated ester BOC-4-aminobenzoic acid NHS ester 47 (82%). Amidation of 47 with pent-4-yn-1-amine 60 produced BOC-protected final product 61 which was deprotected in a mixture of TFA and DCM. Providing the label 36, after column chromatography as a light brown solid in 61% yield.
2.4 Conclusion

In conclusion, this chapter details the successful synthesis of two multifunctional labels for the analysis of glycans. The synthesis of 35 was performed in a linear 5 step reaction with an overall yield of 60.6% through the synthesis of the BOC-protected activated 47 before formation of the amide with the propargyl containing amine. Label 36 was synthesised in a linear 4 step reaction in with an overall yield of 45%. The simple synthesis of these molecules has produced labels with additional functionality that can be used for many further uses by utilising the CuAAC reaction on the alkyne group.

2.5 Experimental

**General experimental**

All chemicals were purchased from commercial sources (Fisher Scientific, Sigma Aldrich, Alfa Aesar, Acros Organics, Fluorochem) and used without further purification. Nuclear Magnetic Resonance (NMR) solvents were purchased from Sigma Aldrich, Fisher Scientific or Fluorochem.

Proton NMR (δ\textsubscript{H}) was recorded on a Bruker Nano400 (400 MHz) spectrometer or a Bruker DPX400 (400 MHz) spectrometer. Proton spectra were assigned using 2D NMR where appropriate. Carbon NMR (δ\textsubscript{C}) were recorded on a Bruker Nano400 (100.7 MHz) spectrometer or a Bruker DPX400 (100.7 MHz) spectrometer and were assigned using heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) when necessary. Residual chemical shifts are quoted on the δ-scale in parts per million (ppm) using residual solvent as internal standard.
Attenuated total reflectance infrared spectroscopy (ATR-IR) was performed on a Perkin-Elmer 100 FTIR spectrometer. Melting points were obtained with a Cole-Parmer™ Stuart™ Digital Melting Point Apparatus and are uncorrected. High resolution mass spectrometry (HRMS) was performed by direct injection mass spectrometry (DI-MS) on a ThermoFisher Scientific Orbitrap XL. Experiments were monitored with thin layer chromatography (TLC). TLCs were carried out on aluminium backed Merck Millipore TLC silica gel 60 F254 plates. Visualisation of TLC plates was accomplished by UV (λ = 254 nm or 302 nm) and either potassium permanganate or ninhydrin.

2.5.1 Synthesis of multi-functional glycan labels

**Boc-4-aminobenzoic acid**

4-aminobenzoic acid 41 (200 mg, 1.46 mmol) was dissolved in water (1.6 mL) and dioxane (3.2 mL). To this NEt₃ (0.26 mL, 1.87 mmol, 1.3 eq) and di-tert-butyl dicarbonate (426 mg, 2 mmol, 1.4 eq) was added in one go and the solution was stirred at RT. After 24 h, the solvent was removed *in vacuo* to leave a viscous colourless oil. HCl 3N (aq) (6 mL) was added in one go and the mixture stirred until a white precipitate had formed. The precipitate was collected via filtration, washed with water (3 x 5 mL) and petroleum ether (3 x 5 mL), then dried to afford pure BOC-4-aminobenzoic acid 46 as a white solid. (294.9 mg, 1.2 mmol, 85%).
Rf EtOAc: petroleum ether (40:60) (2:3) 0.5; MP 192-194°C. 1H NMR (400 MHz, MeOD-d4) δ ppm, 1.50 (s, 9H, 3CH₃), 7.48 (d, J = 8.7 Hz, 2H, 2CH aromatic), 7.89 (d, J = 8.8 Hz, 2H, 2CH aromatic), 9.73 (s, 1H, NH); 13C NMR (101 MHz, DMSO-d6) δ ppm 28.51 (3CH₃), 80.12 (C-(3CH₃), 117.68 (CH aromatic), 124.43 (CH aromatic), 130.82 (CH aromatic), 144.24 (CH aromatic), 153.01 (NCO), 167.48 (-C=O)OH.255; IR (ATR-FTIR) cm⁻¹ 1523 (m, N-H bend), 1657 (s, C=O stretch), 1701 (s, C=O stretch), 3304 (m, O-H stretch), 3420 (m, C-H aromatic stretch), 3481 (m, N-H amide stretch); HRMS (ESI+) C₁₂H₁₅NNaO₄⁺ expected: 260.0899 (M + Na⁺) found: 260.0896 (M + Na⁺).

BOC-4-aminobenzoic acid NHS ester

BOC-4-aminobenzoic acid 46 (200 mg; 0.84 mmol) and NHS (96.8 mg; 0.84 mmol, 1 eq) were dissolved in THF (4.2 mL). To this DCC (173.3 mg, 0.84 mmol, 1 eq) was added and the mixture was stirred at RT overnight. A precipitate of dicyclohexylurea formed that was removed by filtration. The solution was dried under reduced pressure and the resulting white solid was pre-absorbed onto silica and purified by flash chromatography (ethyl acetate : petroleum ether 40 – 60, 1 : 1) to give the pure product as a white solid. (245.1 mg, 0.7336 mmol, 87 %).

Rf EtOAc : petroleum ether 40 – 60, 1 : 1, 0.6. MP 171-172°C. 1H NMR (400 MHz, CDCl₃) δ ppm 1.53 (s, 1H, 3CH₃), 2.90 (s, 4H, 2(CO)CH₂), 6.77 (s, 1H, NH), 7.50 (d, J = 8.8 Hz, 2H, 2CH₂ aromatic), 8.06 (d, J = 8.8 Hz, 2H, 2CH₂ aromatic) 255. 13C NMR (101 MHz, CDCl₃) δ ppm 169.60 (ONCO), 161.53 (ArCOO), 152.08 (HNCO), 144.82 (CH₂ aromatic), 132.28 (CH₂ aromatic,
118.88 (CH$_2$ aromatic), 117.66 (CH$_2$ aromatic), 81.78 (CH(CH$_3$)), 28.42 (3 x CH$_3$), 25.85 (2 x (CO)CH$_2$). $^{13}$C NMR (101 MHz, DMSO-d6) δ ppm 28.51 (3CH$_3$), 80.12 (C-(3CH$_3$), 117.68 (CH aromatic), 124.43 (CH aromatic), 130.82 (CH aromatic), 144.24 (CH aromatic), 153.01 (NCO), 167.48 (COOH) $^{258}$. IR (ATR-FTIR) IR cm$^{-1}$ 1552 (m, N-H bend), 1609 (s, C=O stretch), 1740 (s, C=O stretch), 2963 (m, C-H stretch), 3222 (m, N-H amide stretch); HRMS (ESI+) $^{16}$H$_{38}$N$_2$NaO$_6$ expected: 357.10626 (M+Na$^+$) found: 357.10610 (M+Na$^+$).

4-amino-N-{2-[(ethyl(prop-2-yn-1-yl)amino)ethyl]benzamide 35

![Chemical Structure](image)

Activated ester, BOC-4-aminobenzoic acid NHS ester 47 (200 mg, 0.6 mmol), was dissolved in DCM (8 mL). N-ethylethylenediamine 44 (141.76 µL, 0.7425 mmol) and NEt$_3$ (625 µL, 1.3581 mmol) was then added drop-wise to the solution. The reaction mixture was stirred for 6 h then DCM (16 mL) was added to the reaction mixture, and the solution was extracted with a saturated solution of aqueous sodium bicarbonate (50 mL). The organic phase was collected and dried over magnesium sulphate. The solvent was then evaporated, leaving a solid residue identified as tert-butyl 4-((2-(ethylamino)ethyl)carbamoyl)phenyl)carbamate 48, which was used without further purification.

Crude tert-butyl 4-((2-(ethylamino)ethyl)carbamoyl)phenyl)carbamate 48 (500 mg, 1.5 mmol) was dissolved in MeCN (20 mL). To this potassium carbonate (249 mg, 1.8 mmol, 1.2 eq) was added along with 80% propargyl bromide 49 in THF (357 µL, 1.8
mmol, 1.2 eq). This solution was stirred at RT. After 48 h the reaction was concentrated in vacuo. DCM (20 mL) was added, and the solution was filtered to remove potassium carbonate. The protected crude product was purified by flash chromatography (DCM: MeOH, 9:1) and the following orange oil was deprotected in DCM : TFA (3:2). The deprotection was monitored by TLC until the spot corresponding to the protected species had disappeared. After this, the solvent was removed in vacuo and residue was dissolved in a minimum of MeOH. The solution was made neutral with the addition of amberlite, the solution was stirred at RT until pH 6-7. Amberlite was removed by filtration and the solvent was evaporated under reduced pressure to yield 4-amino-N-(pent-4-yn-1-yl)benzamide 35 as a thick dark yellow oil. (301 mg, 1.23 mmol, 82%).

**Rf** EtOAc : Petroleum ether 40-60, 3 : 2, 0.3. **1H NMR** (400 MHz, CDCl3) δ ppm 1.09 (t, J = 7.0 Hz, 3H, NCH2CH3), 2.22 (s, 1H, C≡C-H ), 2.63 (q, J = 6.7 Hz, 2H, NCH2), 2.77 (t, J = 5.8 Hz, 2H, NCH2), 3.46 (s, 2H, NCH2C≡C), 3.50 (q, J = 4.9 Hz, 2H, NCH2CH3), 4.04 (s, 2H, NH2), 6.63 (d, J = 8.0 Hz, 2H, aromatic CH), 6.76 (s, 1H, NHCO), 7.61 (d, J = 8.0 Hz, 2H, aromatic CH). **13C NMR** 101 MHz, CDCl3) δ ppm 12.57 (CH2CH3), 36.79 (NCH2C≡C ), 41.32 (NCH2CH2), 47.49 (NCH2CH3), 51.95 (NCH2), 73.58 (C≡C-H ), 77.84 (CH2C≡C-H), 114.10 (aromatic CH), 123.98 (aromatic CH-CO), 128.71 (aromatic CH), 149.62 (aromatic C-NH2), 167.27 (CONH); IR (ATR-FTIR) IR cm⁻¹ 3337 (m, N-H stretch), 3226 (m, N-H stretch), 2970 (m, C-H stretch), 2923 (m, C-H stretch) 2849 (m, C-H stretch ) 2123 (w, alkyne stretch) 1602 (s, C=O stretch) 1541 (m, N-H bend) 1503 (s, C-C aromatic bend); **HRMS** (ESI-MS) C14H20N3O+ expected: 246.1601 (M+H⁺) found: 246.1603 (M+H⁺). C14H19N3NaO+ expected: 268.1420 (M+Na⁺) found: 268.1426 (M+Na⁺).

4-amino-N-(pent-4-yn-1-yl)benzamide 36

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BOC-4-aminobenzoic acid NHS ester 47 (200 mg, 0.599 mmol) was dissolved in DCM (8 mL). 4-pentyn-1-amine hydrogen chloride 60 (160 mg, 1.34 mmol, 2.25 eq) and NEt₃ (188 µL, 1.36 mmol, 2.25 eq) was added dropwise to the solution. The reaction mixture was stirred for 16 h then solvent was removed in vacuo. The crude protected product was purified by column chromatography (ethyl acetate : petroleum ether 40–60, 1:1).

The resulting solid was deprotected in a mixture of DCM : TFA (5 mL, 3:2). The deprotection reaction was monitored by TLC (DCM : MeOH, 9:1) with UV visualisation. DCM was removed under reduced pressure once the spot corresponding to the protected product had disappeared and MeOH (20 mL) was added. Amberlite (2 g) was added and left to stir in solution at RT. Once the solution reached neutral pH, resin was removed by filtration and solvent was removed in vacuo. The resulting crude product was purified by column chromatography (DCM : MeOH, 9:1) yielding pure glycan label 36 as a light brown solid. (72 mg, 0.36 mmol, 61%).

Rᵥ DCM:MeOH, 9:1, 0.7. MP 98 – 100 °C. ¹H NMR (400 MHz, MeOD₄) δ ppm 1.87 – 1.71 (m, 2H, CH₂CH₂CH₂), 2.31 – 2.18 (m, 3H, C≡C-H and CH₂CH₂C≡C), 3.42 (t, J = 6.7 Hz, 2H, NHCH₂CH₂), 6.66 (d, J = 8.3 Hz, 2H, aromatic CH), 7.59 (d, J = 8.3 Hz, 2H, aromatic CH). ¹³C NMR (101 MHz, MeOD₄) δ ppm 16.8 (CH₂CH₂CH₂), 29.7 (CH₂CH₂C≡C), 40.0 (NHCH₂), 69.9 (C≡C-H), 84.4 (C≡C-H), 114.7 x 2 (aromatic C-H), 123.3 (aromatic C=C=O), 129.9 x 2 (aromatic C-H), 153.0 (aromatic C-NH₂), 170.5 (C=O). IR (ATR-FTIR) IR cm⁻¹ 1289 (s, C-O stretch), 1507 (m, N-H bend), 1613 (s, C=O)
stretch), 2927 (m, C-H alkane stretch), 3235 (m, C-H aromatic stretch) 3325 (m, C-H alkyne stretch) 3440 (m, N-H stretch); HRMS (ESI+) C_{12}H_{14}N_{2}NaO^{+} expected: 225.0998 (M + Na^{+}) found: 260.0896 (M + Na^{+}).
Chapter 3 - The analysis of glycans bearing multifunctional labels

3.1 Background

3.1.1 HILIC-HPLC analytical workflow of 35 and 36 labelled glycans

The multifunctional alkyne labels 35 and 36 synthesised in Chapter 2 (Figure 34) bear alkyne groups which may undergo selective reactions with azides and may increase detection sensitivity in subsequent MS analysis. These labels also retain the conjugated aromatic region and amine group that are required for optical detection of the labelled glycan and covalent linking to the glycan respectively which are crucial for HPLC-FLD analysis. In this prospective workflow, glycans will be enzymatically released by PNGase F, derivatised with label 35 or 36, purified by SPE and collected after separation and detection by HILIC. These collected glycans will then be conjugated with an azide conjugation partner. This azide conjugation reaction are aimed to facilitate downstream analysis, in this case with MS or FLD of the conjugated labelled glycan to increase detection sensitivity.

![Figure 34: Structures of multifunctional labels 35 and 36.](image)

Greater understanding of the mechanisms behind the ionisation of analyte in these downstream detection techniques provides the ability to synthesise azides that will ensure an increase in detection sensitivity for the triazole product of the labelled glycan.

3.1.2 Analyte ionisation in ESI-MS
The ionisation of an analyte with ESI occurs when a voltage is applied to droplets containing analyte produced by a spray atomiser. These charged analytes are then liberated from the droplet into the gaseous phase where they are detected. However, the mechanism causing the liberation of the charged ion is debated with three models having been proposed to explain it. These models are the chain ejection model (CEM), the ion evaporation model (IEM) and the charge residue model (CRM).

The IEM was proposed by Iribarne and Thomson (1976) to explain the generation of atomic ions from randomly charged droplets and the CRM was proposed by Dole et al. (1968) stating that droplets are formed containing only one analytical ion. The CEM was proposed by Konermann et al. (2012) to account for the ionisation of proteins when analysed under non-native conditions. The IEM and CRM represent the cornerstone for any discussion related to the process of ESI.

The IEM proposes droplets are produced containing hundreds of charged ions, as these droplets shrink to approximately 10 nanometres in size by evaporation, the field strength at the surface of the droplet is large enough to expel the solvated ions from the droplet. In this model, the rate of expulsion is heavily influenced by the chemical properties of the ion such as the enthalpy differences that needs to be overcome. An example of this expulsion is shown in the top route of Figure 35. However, the IEM is limited as it does not account for different evaporation rates encountered for different ions and why ions with very different solvation energies have similar ionisation rate constants.
Figure 35: A representation for the mechanisms behind ion formation in both the IEM and CRM. In the IEM (top path) the droplet contains multiple charged ion, as the size of the droplet reduces by evaporation the increase in field strength at the surface of the droplet expels the ions. In the CRM (the bottom path) the ion is released through evaporation of the droplet.\textsuperscript{261,263}

The CRM model proposes that ions are liberated into the gaseous phase from very small droplets containing one analytical ion assumed to have been formed by a succession of Rayleigh disintegrations.\textsuperscript{262} This route of expulsion is shown in the bottom route of Figure 35. In this model, ionization is strongly dependent on the efficiency of solvent evaporation determined primarily by the ion current and not the physicochemical properties or mass of the analyte.\textsuperscript{263}

IEM does not account for why ions with very different solvation energies have similar ionisation rate constants or why different evaporation rates are encountered for different ions. CRM does not account for the observation that ionisation is benefitted by the hydrophobicity of the analyte which is contradictory to this model.\textsuperscript{263}

Though the exact mechanism behind ionisation is not fully understood, successful efforts have been made to improve the detection of analyte in MS. The observation that the hydrophobicity of a molecule contributes to increased ionisation has been used to increase detection sensitivity of glycan labels. Hunter Walker \textit{et al} (2011)
produced a library of hydrazide labels 62 – 66 (Figure 36) with increasing hydrophobicity aimed at increasing the ionisation efficiency and detection sensitivity of labelled carbohydrates in MS \(^{264}\). Hydrophobicity was increased through the introduction of additional phenyl groups or by the elongation of the alkyl chain \(^{264}\). This research illustrated that increased hydrophobicity of the label led to increased detection sensitivity of the analyte in MS \(^{264}\).

![Figure 36: Structure of hydrazides 62-66 from Hunter Walker et al (2011).](image)

These observations present a clear method to increase the hydrophobicity of the labelled glycans after azide conjugation. This could be done by conjugating hydrophobic azides to the alkyne group of the labelled glycan to produce a hydrophobic conjugated product. This in turn will increase the detection sensitivity of the conjugated labelled carbohydrates in off-line detection methods. These hydrazides created by Hunter Walker et al (2011) give a basis for the synthesis of hydrophobic azides. Azide equivalents of these hydrazides would be synthesized to increase the hydrophobicity of the azide conjugated labelled glycans to improve MS sensitivity.

### 3.1.3 Analyte detection in FLD detection

The addition of the azide conjugation partner was also aimed to increase the detection sensitivity of the azide conjugated labelled glycan in subsequent FLD analyses. The absorbance of a photon with a fluorophore can be explained by the Beer-Lambert’s Law \((A = \varepsilon cd)\), where \(A\) is absorbance, \(\varepsilon\) is the molar absorption
coefficient, c is concentration and d is path length, the distance the light passes through the solution. A commonly used method of increasing the molar absorption coefficient is to increase the degree of conjugation in the molecule $^{265}$. Increasing the conjugation of a molecule decreases the energy gap for the $\pi - \pi^*$ transition between the HOMO and LUMO and increases molar absorption efficiency $^{266}$. This observation presents a clear method for increasing the FLD detection sensitivity of the labelled glycan by reacting to a conjugated azide conjugation partner.

3.2 Aims

The work in this chapter had two main aims. The first was to evaluate whether novel multifunctional labels $^{35}$ and $^{36}$ could be employed as glycan labels in a glycan analysis workflow. This was examined by labelling of oligosaccharides from a variety of sources and analysing these samples in HILIC mode HPLC with FLD detection. These analyses would be compared to the analysis of ProA $^{21}$ labelled oligosaccharides from the same sources.

This chapter will also synthesise a series of azides which will react with the alkyne group of the multifunctional labels post glycan analysis. These azides are aimed to increase the hydrophobicity of the azide conjugated labelled carbohydrate to increase detection sensitivity in MS. Three azides, (Figure 37) benzyl azide $^{33}$, (3-azidopropyl)benzene $^{67}$ and (3-azidobutyl)benzene $^{68}$, based on the hydrazine labels 2-phenylacetohydrazide $^{62}$, 3-phenylpropanehydrazide $^{64}$ and 4-phenylbutanehydrazide $^{65}$ produced by Hunter Walker et al (2011) would be used $^{264}$.  

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In addition, the synthesis of a further two azide molecules (Figure 38) containing large hydrophobic π-systems based on the glycan label 6-AQ 17, aimed to increase molar extinction coefficient and fluorescence quantum yield of the resulting triazole product, was also performed. The first of these azides, 6-azidoquinoline 69, is a derivative of 6-AQ 17. The second, the charged azidoquinolinium 70 is formed by the methylation of 69. The large hydrophobic quinoline group in 6-AQ 17 is partly responsible for the MS ionisation and fluorescent properties of RF-MS 26 making it an ideal parent compound for the synthesis of a hydrophobic and fluorescent azide 158, 267.

The methods used to fulfil the aims from this section are summarised in the flowchart in Figure 39 which details the experiments, types of glycans and labels employed in this Chapter.
Figure 39: A flowchart showing the prospective order of research in this Chapter highlighting the methods, labels and glycans employed.
3.3 Results and discussion

The first aim of this chapter was to investigate whether the analysis of glycans labelled with the multifunctional labels resulted in similar detection sensitivity, accuracy and resolution to the labels used currently. Establishing that these labels produce adequate separation and precise and sensitive detection of the analyte is necessary to ensure accurate and precise analysis in glycan analysis workflows. The analysis of these samples was to be performed by HILIC-FLD. Detection in HILIC-FLD relies on the $\lambda_{\text{ex}}$ and $\lambda_{\text{em}}$ of the label to be known as these parameters are defined in the fluorescence detector.

3.3.1 HILIC-HPLC analysis of 21, 35 and 36 labelled carbohydrates

3.3.1.1 Fluorescence of multifunctional labels 35 and 36 labelled maltooligosaccharide

The $\lambda_{\text{ex}}$ Max and $\lambda_{\text{em}}$ Max of the novel labels were determined by fluorescence spectroscopy on a sample of labelled maltoolentaose, an $\alpha-1,4$-linked 5-glucose maltooligosaccharide. The reductive amination labelling reaction of maltoolentaose was performed with labels 35 as well as the control 21 at 65 °C for 3 h in borate-acetate MeOH solution with sodium cyanoborohydride as the reducing agent. A borate-acetate MeOH solution was selected rather than the more common DMSO – acetic acid solution as the acetate-borate MeOH system is more basic at a pH of 5 and should reduce the loss of sialic acid in sialylated samples related to the acidic conditions.  

The labelled maltoolentaose samples were purified by SPE with Spe-ed2 amide-SPE (Applied Separations Inc.) cartridges and purified samples were dried. The dried samples were reconstituted to produce a final solution at 80 pmolµL$^{-1}$ in MeCN: 50 mM ammonium formate pH 4.4 (aq) (65 : 35). The samples were reconstituted in this
solvent system to achieve closer representation of the solvent environment during the HILIC analysis that would be used later, as the fluorescence properties of a molecule, including $\lambda_{ex}$ and $\lambda_{em}$ wavelengths, can change depending on chemical environment including dissolution solvent 269.

500 µL of both the 21 and 35 labelled maltopentaose solution was analysed by 3D fluorescence spectroscopy to ascertain approximate $\lambda_{ex}$ Max and $\lambda_{em}$ Max values. In this experiment, 3D fluorescence spectroscopy of the labelled maltopentaose was excited between 250 nm and 500 nm using 10 nm increments. Emission was recorded between 300 nm and 600 nm. The $\lambda_{ex}$ Max and $\lambda_{em}$ Max of label 36 was established by other group members so 3D fluorometry was not required. Figure 40 shows the 3D emission scans for both 21 and 35 labelled maltopentaose.
The overlaid emission scans in the Figure 40 shows the emission wavelengths at each excitation wavelength. Comparing Figure 40 (a) and Figure 40 (b) the $\lambda_{\text{ex Max}}$ of both labels were between 290 and 300 nm and the $\lambda_{\text{em Max}}$ was between 350 nm and 375 nm. These wavelengths were used to determine the $\lambda_{\text{ex Max}}$ and $\lambda_{\text{em Max}}$ of the control, commercially available glycan label 21 and the terminal alkyne
derivative 35 in single wavelength fluorescence scans. **Figure 41** shows the overlaid excitation and emission spectra of maltopentaose labelled with 21, 35 and 36.

**Figure 41:** Fluorescence spectra of maltopentaose labelled with a) 21 b) 35 and c) 36.
In Figure 41 (a), the optimal fluorescent wavelengths for maltopentaose labelled with 21 were found at $\lambda_{\text{ex}} = 299$ nm and $\lambda_{\text{em}} = 367$ nm (c.f. literature values: $\lambda_{\text{ex}} = 310$ nm and $\lambda_{\text{em}} = 370$ nm for labelled IgG glycans.) The optimal wavelengths for label 35 shown in Figure 41 (b) were determined to be $\lambda_{\text{ex}} = 296$ nm and $\lambda_{\text{em}} = 361$ nm. As both 21 and 35 share a number of structural similarities, the similarities between the $\lambda_{\text{ex}}$ Max and $\lambda_{\text{em}}$ Max values of the two labels were expected. As 36 displays structural differences to 21 and 35 it was expected that label 36 would differ in their fluorescence properties. Figure 41 (c) illustrates this, as the fluorescence of maltopentaose labelled with 36 had $\lambda_{\text{ex}}$ Max and $\lambda_{\text{em}}$ Max values of 281 nm and 346 nm, a slight difference to labels 21 and 35.

3.3.1.2 Determining the lower limit of detection of 21, 35 and 36 labelled maltopentaose

After determining the fluorescence parameters of the labels, the detection sensitivity of the labels could then be compared. When synthesising the glycan label derivatives, it was aimed that these analogues would display a similar lower limit of detection (LLOD) to the parent compounds. A reduction in detection sensitivity may prevent the detection of low abundance analytes and may reduce the potential adoption of the derivative for glycan analysis. Alternatively, if the derivative has improved detection sensitivity meaning that analytes are detectable at lower abundance it may allow the detection of species not possible with currently used labels.

The LLOD of these labels were investigated by HILIC-HPLC-FLD analysis of maltopentaose labelled with 35 and ProA 21. Both samples were prepared in identical fashion, 500 pmol of maltopentaose was labelled with the same amount of each label in acetate-borate MeOH solution with sodium cyanoborohydride at for 3 h at 65 °C. The labelling solution was purified by amide SPE, the purified samples were dried and reconstituted in water before being diluted further as shown in Table 4.
### Table 4: Dilution of labelled maltopentaose standards.

<table>
<thead>
<tr>
<th>Final concentration</th>
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<tbody>
<tr>
<td>50 pmol mL⁻¹</td>
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<tr>
<td>25 pmol mL⁻¹</td>
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<tr>
<td>10 pmol mL⁻¹</td>
</tr>
<tr>
<td>5 pmol mL⁻¹</td>
</tr>
<tr>
<td>2.5 pmol mL⁻¹ (from 25 pmol mL⁻¹ solution)</td>
</tr>
<tr>
<td>1 pmol mL⁻¹ (from 25 pmol mL⁻¹ solution)</td>
</tr>
</tbody>
</table>

1 μL of each solutions was injected for HPLC-FLD analysis from low to high concentration. HPLC was performed on an Agilent 110 series HPLC system with an Agilent 1100 series G1321A FLD detector. Separation was performed on a TSKgel® Amide-80 HR (25 cm × 4.6 mm, 5 μm particle size) column (TOSOH Bioscience) with a TSK® guardgel Amide-80 (1.5cm × 3.2 mm, 5 μm particle size). (TOSOH Bioscience) The mobile phase consisted of ammonium formate (aq) (50 mM, pH 4.4) as solvent A and MeCN composing solvent B. The FLD traces of these injections are shown in Figure 42.
Figure 42: LLOD of maltopentaose labelled with ProA 21 (a) and derivative 35 (b).

There are a number of similarities between the traces in Figure 42 (a) and Figure 42 (b). The concentration where the analyte is no longer discernible from the baseline for both labels was 10 pmolmL\(^{-1}\), this is an equivalent to a 10 femtomole injection of analyte. As no notable change in the LLOD is observed it suggests that 35 has similar detection sensitivity to the control label 21. The LLOD of detection for label 36 was determined by other group members (not shown).

3.3.1.3 Standard calibration curve of 21, 35 and 36 labelled maltopentaose

Once the LLOD for labelled 35 samples were deemed similar to parent compound 21, a standard calibration curve of carbohydrate labelled with 21 and 35 was produced. Standard calibration curves are regression models commonly used to quantify analytes in HILIC \(^{270}\). The detector response of an analyte at unknown concentration is compared to the response produced by a set of linear standards at known concentrations. The quantitative analysis of glycans is common during the quality
control of biotherapeutic production to ensure the concentrations of glycans on therapeutic proteins are at safe ranges $^{121, 174}$.

Labels 21 and 35 were labelled with lactose, the labelled carbohydrates were purified by SPE then dried. The dried analyte was weighed and dissolved to a stock solution at a final concentration of 100 μM, this stock solution was diluted to 75 μM, 50 μM, 25 μM, 10 μM, 5 μM and 0 μM. To produce the standard calibration curve, 1 μL of each solution was injected in triplicate by HILIC-FLD starting at lowest concentration. The overlaid responses of these injections and the plot of peak area versus concentration is shown in Figure 43.
Figure 43: Evaluation of the linearity of labelled lactose standard for quantitative analysis. a) shows the triplicate injections of ProA 21 labelled lactose between 100 µM and 0 µM. b) shows the triplicate injections of 35 labelled lactose between 100 µM and 0 µM. c) shows the plot of the peak area for each of 21 labelled lactose concentrations and plot and d) shows the plot of the peak area for each of 32 labelled lactose concentrations. The error bars show a graphical representation of error at each measurement.

The overlaid FLD traces in Figure 43 (a) and Figure 43 (b) show low degree of variation in retention for analyte labelled with labels 21 and 35 as concentration increased. The plot of peak area shown in Figure 43 (c) and Figure 43 (d) indicated high linearity for both labels 21 and 35 in this system. The linearity of an analyte in a system can be explained as the proportional relationship between the concentration and detection intensity of an analyte. Linearity can be graphically represented by a straight line and by calculating the coefficient of determination for the values.

The coefficient of determination ($r^2$) is a statistical measure in a regression model that determines the proportion of variance. It can be used to analyse how the change in one variable can be explained by a difference in a second variable. The value of $r^2$ lies between 0 and 1, an $r^2$ closer to 1 indicates a better goodness of fit to the linear regression. In this case, the $r^2$ values for the linear regression of peak area for labels 21 and 35 in Figure 43 (c) and Figure 43 (d) displayed satisfactory coefficient of
determination as both were close to 1. The 35 labelled samples had a slightly higher $r^2$ value at 0.9999 compared to 0.9997 for the ProA 21 labelled species.

3.3.1.4 HILIC-HPLC-FLD analysis of 21, 35 and 36 labelled GHP.

After confirming that linearity and LLOD for label 21 and 35 were satisfactory, the analysis of glycans labelled with the novel labels 35 and 36 was then performed. This first analysis was performed with a sample of labelled GHP. Labelled GHP is used as retention standards, the retention of an unknown glycan is compared to the retention of the GHP standards to elucidate structure 273. GHP was selected as this analyte will demonstrate whether the separation and detection of the novel labels 35 and 36 is comparable to ProA 21.

The GHP was produced from the hydrolysis of dextran, a branched glucan with a molecular mass of ~200,000 consisting of mainly of α-1,6-linked glucose from *Leuconostoc mesenteroides*. Dextran was hydrolysed in dilute hydrochloric acid for 4 h at 100 °C. This solution was labelled with labels 35 and 36 and 21 in a borate-acetate MeOH solution with sodium cyanoborohydride at 65 °C for 3 h. The samples were purified on spe-ed2 amide (Applied Separations Inc.) SPE cartridges, purified samples were dried in a vacuum centrifuge concentrator and dried samples were stored at -20 °C before reconstituting in water prior to injection.

These samples were analysed by HPLC-FLD with the detector set to the wavelengths obtained from fluorescence spectroscopy. A sample of ProA 21 labelled sample were also analysed with the fluorescence wavelengths used by Kozak et al (2015) ($\lambda_{ex} = 310$ nm and $\lambda_{em} = 370$ nm) to see which resulted in the highest detection sensitivity 274. Figure 44 shows the HPLC-FLD traces of GHP labelled with 21, 35 and 36. For this analysis a detector gain of 10 was used. Gain is a parameter on fluorescence detectors that adjusts the response of a detector by controlling the voltage across a photomultiplier tube.
Figure 44: HILIC-FLD analysis of GHP labelled with a) ProA 21 with wavelengths collected by fluorescence spectroscopy, b) ProA 21 with wavelengths from literature sources c) label 35 and d) label 36. While only one injection is shown for each label in this figure, during this research this analysis was performed in excess of 100 times with highly similar results.
The chromatograms in Figure 44 all display similar for all samples, the order of elution for each sample is consistent with the low glucose units (GU) species eluting first as had been expected. The FLD trace in Figure 44 (a) is the result of the injection with the wavelengths determined experimentally whereas Figure 44 (b) is the result of the injection with the fluorescent wavelengths from literature sources 270. Figure 44 (a) shows a slight increase in signal intensity compared to Figure 44 (b), this is observed for the peak corresponding to labelled 1-GU species eluting at approximately 10 mins. In Figure 44 (a) this peak had a signal intensity of 100 compared to 82 for the same peak in Figure 44 (b) using the fluorescent wavelengths stated by Kozak et al (2015). Comparing the detection sensitivity clearly indicates the wavelengths collected experimentally result in greater sensitivity, therefore, these wavelengths were used solely in future HPLC-FLD analysis of ProA 21 labelled samples.

Figure 44 (c) shows the analysis of the 35 labelled GHP sample, this trace the peak corresponding to the labelled GU1 species at 103 intensity which is similar to the detection intensity of the ProA 21 labelled species in Figure 44 (a) further confirming the similarity of the detection sensitivity for both labels.

The small structural change between the two labels was expected to not result in a notable change in retention particularly as retention in HILIC is driven by the hydrophilic interactions of the carbohydrate 275. From Figure 44 (c) the 35 labelled analytes eluted approximately one minute earlier than samples labelled with the parent compound 21 which was unexpected. In total, more than thirty GU could be identified from Figure 44 (c) before further assignment was not possible. This is similar to ProA 21 labelled species where thirty GU could also be identified.

Analysis of 36 labelled GHP in Figure 44 (d), showed the peak corresponding to the labelled GU1 species had a signal intensity of approximately 120, a detection higher than both labels 21 and 35 in Figure 44 (b) and Figure 344 (c). This label also resulted in the earliest elution of the GU1 species for any of the three labels, with the first
peak eluting approximately 5 mins earlier than ProA 21 and 35 labelled samples in Figure 44 (d) indicating that label 36 is the least hydrophilic of the three labels. Only twenty peaks corresponding to 36 labelled GHP could be identified in Figure 44 (d), fewer than was possible in Figure 44 (b) and Figure 44 (c).

The relation between GU and elution time for the first 20 GU from Figure 44 (b), Figure 44 (c) and Figure 44 (d) was plotted. These plots may be used to elucidate the structure of unknown glycans by comparing the retention time of an unknown glycan to retention time of a labelled GHP. The unknown glycans are given a representative GU value to estimate the structure of the glycan. The plot of relation between GU and retention time for GHP labelled for each of the three labels is shown in Figure 45.
Figure 45: Plot of the relationship between GU and time of elution for GHP labelled with a) ProA 21, b) ProA alkyne derivative 35 and c) label 36.
For each plot, the relationship between retention time and GU was expressed through the calculation of a coefficient of determination. The coefficient of determination for the plot of all of the labels in Figure 45 is high indicating high agreement to the line of best fit. The ProA 21 labelled sample in Figure 45 (a) had a $r^2$ value of 0.9995 whereas the relation between retention time and GU for 35 labelled species in Figure 45 (b) was slightly lower with an $r^2$ value of 0.9988 with non-linear regression curve fitting. The lowest coefficient of determination of the three labels was seen for the 36 labelled species in Figure 45 (c) at 0.9950 with a linear regression curve fitting. These values however all close to 1 illustrating the similarities for each of these labels.

3.3.1.5 HILIC-HPLC-FLD analysis of 21, 35 and 36 labelled RNase B glycans

The effective separation and detection of the novel labels 35 and 36 in the analysis of the labelled GHP samples clearly demonstrated the ability of these labels in analysis of oligosaccharides. Further analysis with labels 35 and 36 was performed for glycans from natural sources. The obvious choice for these native glycans were glycans from bovine pancreatic ribonuclease B (RNase B). RNase B is a small (17 kDa) glycosylated protein; the oligomannose glycans that RNase B displays are well characterised making RNase B glycans ideal for the analysis of natural glycans with multifunctional labels 35 and 36 \cite{276,277}.

The release of RNase B glycans was performed enzymatically with recombinantly expressed PNGase F produced in house. Deglycosylation was performed under denaturing conditions with a solution of 0.5% SDS and 50 mM dithiothreitol at 95 °C. The enzymatically released glycans were purified by SPE with Hypercarb Hypersep PGC SPE (Thermo Scientific) cartridges. The purified and dried released glycan were labelled in acetate-borate MeOH solution with sodium cyanoborohydride in a thermomixer at 65 °C for 3 h, labelled glycans were purified by SPE on a spe-ed2 amide cartridge (Applied Separations Inc.). Purified glycans were reconstituted in H2O and 1 μL of the 21, 35 or 36 labelled RNase B glycan solution was analysis by HILIC-FLD. Due to the lower abundance of the analyte
compared to GHP, a higher gain of 12 was used. The FLD traces of these injections are shown in Figure 46.

Figure 46: HILIC-FLD trace of RNase B glycans labelled with a) ProA 21 and b) ProA derivative 35 and c) label 36. Structures were assigned by comparison to literature 278. While only one injection is shown for each label in this figure, during this research this analysis was performed in excess of 50 times with highly similar results.
All of the traces in Figure 46 share similarities, each trace shows the five peaks representing one of the five oligomannose glycans that are characteristic of the analysis of the RNase B glycome \(^{279}\). Labels 35 and 36 clearly show similar results to the commonly used label ProA 21 with all traces showing the same elution pattern. The change in retention time for each label that had been observed in Figure 44 for the analysis of the labelled GHP samples is also present with these samples. The 35 labelled sample eluted approximately 3 mins earlier than the ProA 21 labelled samples and the 36 labelled samples which eluted the earliest in Figure 44 (c) eluted considerably earlier in this analysis, 9 mins earlier than the ProA 21 labelled samples in Figure 46 (c).

The highest detection sensitivity was observed for the 21 labelled glycans in Figure 46 (a) with the labelled Man\(_5\) glycan at an intensity of 52 whereas the 35 labelled Man\(_5\) glycan in Figure 46 (b) was the next most intense at 40. The 36 labelled species produced the lowest signal for the labelled Man\(_5\) species at 24 in Figure 46 (c) which is in contrast to what was observed in Figure 44 (c) where 36 displayed the highest detection sensitivity in the analysis of the GHP labelled sample.

From Figure 46, all labels produced similar resolution. Furthermore and the differences in signal intensity observed for each of the labels made it possible to detect and assign each of the RNase B glycans.

3.3.1.6 HILIC-HPLC-FLD analysis of 21, 35 and 36 labelled IgG glycans.

After confirming that these novel labels could be used effectively in the detection of glycans from natural sources, the analysis of a pool of more complicated glycans was analysed with each of the labels. In this analysis, the diverse pool of glycans were from human immunoglobulin G (IgG). IgGs are glycosylated mAbs and are common as biotherapeutic agents \(^{280}\). As IgG glycans are commonly analysed for quality control processes in biotherapeutic mAb production the IgG glycome is well characterised\(^ {281}\).
The IgG glycans were released enzymatically and purified with the same method used in the release and purification of the RNase B glycans. IgG glycans were reconstituted in water and 1 µL of this solution was injected for HILIC-FLD analysis. The FLD trace of the injection from this labelling with ProA 21 is shown in Figure 47. The analysis of all IgG labelled species was performed with a gain of 12.

Figure 47: HILIC-FLD traces of ProA 21 labelled IgG. The top trace shows the analysis of the 21 labelled IgG glycans produced in house showing the loss of sialylation. The bottom trace is figure produced by Keser et al (2018) reproduced with permission 152.

Desialylation can be seen clearly in the top trace of Figure 47 when compared to the lower trace. The absence of the sialylated A1F glycan and an increase G2F peak at 31 mins due to the loss of Neu5Ac 8 from A1F. Previous research in this area had shown that some desialylation is expected during the labelling reaction, however the use of borate-acetate MeOH solution was hoped to prevent Neu5Ac 8 loss 143, 282.
Attempts to prevent loss of sialic acid in the literature have focused on changing the length or the temperature of the labelling reaction. Anumula et al (1998) performed the analysis of 2-AA 13 labelled fetuin glycans in borate-acetate MeOH solution at pH 5 for 1 hr at 80 °C and commented that under these conditions that a negligible loss of sialic acid was observed 268. Shuang Yang et al (2017) investigated the acid catalysed loss of Neu5Ac 5 from the A2F glycan during labelling in DMSO-acetic acid at 37 °C and 65 °C. At 37 °C, the relative abundance of A2F glycan was 150% higher than the A2F glycan labelled at 65 °C. The relative abundance of the A1F glycan was three-fold higher at 65 °C than at 37 °C and relative abundance of G2F was two-fold higher at 65 °C than at 37 °C indicating that desialylation is more common at higher temperatures 283.

A ProA 21 labelling of IgG glycans was performed at lower temperature aimed to reduce loss of Neu5Ac 8 from sialylated IgG glycans during the labelling reaction. This labelling reaction was performed at 25 °C for 3 h however, the HPLC-FLD trace (not shown) of this injection showed no peaks corresponding to any labelled IgG glycan. There are two possible explanations for these results, either 25 °C is too low a temperature for labelling to occur or a longer labelling time is required.

As the low temperature labelling did not result in addition of the label, the labelling reaction was modified to higher temperature and instead performed at 65 °C. It was further modified taking place in a water bath rather than a thermomixer that had been used until this point. The water bath was hoped to prevent ‘hot spotting’ that occurs when labelling in a thermomixer and in turn reduce the loss of Neu5Ac 8. The product of the 21, 35 and 36 labelling with this method was injected. The FLD trace of these injections are shown in Figure 48.
Figure 48: HILIC-FLD traces of IgG glycans labelled with a) ProA 21, b) 35 and c) 36. Structures were assigned by comparing to HILIC analysis of IgG glycans performed by Keser et al (2018). While only one injection is shown for each label in this figure, during this research this analysis was performed in excess of 20 times with highly similar results.
This modification of the labelling procedure reduced desialylation and the labelling reaction for all carbohydrates was transferred to a water bath. The peaks corresponding to the most abundant glycans, the G0F, G1F, G2F and A1F glycans are labelled in each trace. All of the traces in Figure 48 clearly resemble each other indicating that the novel labels 35 and 36 result in similar analysis to 21. Labels 21 and 35 displayed similar detection sensitivity with the peaks corresponding to the labelled G0F and G1F species in Figure 48 (a) and Figure 48 (b) at intensities of 64 and 62 respectively. The lowest detection sensitivity was from label 36 in Figure 48 (c) which is consistent to the RNase B glycan sample.

The analysis in Figure 48 (b) showed higher resolution than Figure 48 (a). This was observed in the distinct shoulder in the peak corresponding to the labelled G2F species at 29 minutes, whereas in Figure 48 (a) this is a single coeluting peak at 31 minutes. The trace in Figure 48 (c) illustrated further resolution of this peak as the shoulder in Figure 48 (b) was now a new more resolved peak showing the resolution of the 36 labelled species in this HPLC-FLD method was the highest.

Together, the analyses of the labelled glycose homopolymer in Figure 44, the analysis of the labelled RNase B glycans in Figure 46 and the labelled IgG glycans in Figure 48 illustrate the clear similarities of these labels to control label 21. The use of labels 35 and 36 in the analysis of carbohydrates allowed clear detection and assignment in all of the carbohydrate samples. Of the two novel labels, 35 was observed to show the greatest similarity in terms of detection sensitivity and analyte retention. The evidence from this analysis suggest that these multifunctional labels could be employed as glycan labels in some current glycan analysis workflows.

3.3.2 CuAAC conjugation of labelled glycans post HILIC

3.3.2.1 Synthesis of azide conjugation partners
As clear evidence of the ability for these multifunctional labels in glycan analysis was demonstrated, the next aim of this chapter was to produce azides containing molecules that could be reacted with the terminal alkyne present on the label of the collected labelled glycans to increase the detection sensitivity of the analytes in subsequent analysis.

Azide synthesis is commonly performed by the conversion of a primary amine with trifluoromethanesulfonfyl azide, also known as triflic azide, however it is both explosive and hazardous due to the toxic triflic anhydride that is used in its synthesis. Goddard-Borger and Stick (2007) produced imidazole-1-sulfonyl azide hydrogen sulfate (Stick reagent) 75 as a safer azidation agent alternative. Potter et al (2016) further developed the synthesis of Stick reagent 75 into a synthetic route that did not require explosive intermediates. The synthesis of Stick reagent 75 developed by Potter et al (2016) is shown in Scheme 30. This method was used to synthesise Stick reagent 75 for use in later diazotransfer reactions.

Scheme 30: The synthesis of Stick reagent 75 developed by Potter et al. \textit{Reagents and conditions:} a-b) MeCN, 0 °C, 3 h; c) MeCN, 0 °C to RT, 17 h; d) EtOAc/sat NaHCO₃ (aq), 0 °C, 20 mins; e-f) EtOAc, 0 °C to RT, 30 mins (77%).

Imidazole-1-sulfonyl azide hydrogen sulfate 75 was synthesised successfully in 77% yield. 75 was then applied in the synthesis of azides 67, 68, 69 and 70 through the azide transfer reaction converting an amine group to the azide. First, 75 was used in the azide conversion of the primary amine 3-phenylpropan-1-amine 76 using a
general method of azide conversion developed by Goddard-Borger and Stick (2007)\textsuperscript{286}. The diazotransfer of \( \text{76} \) is shown in Scheme 31.

![Scheme 31](image)

**Scheme 31**: Azide transfer reaction of 3-phenylpropan-1-amine \( \text{76} \) with Stick reagent \( \text{75} \) using a method developed by Goddard-Borger and Stick\textsuperscript{286}.

Stick reagent \( \text{75} \) in 1.2 eq excess was combined with a 1 mol\% aqueous solution of CuSO\(_4\) and 2 eq of K\(_2\)CO\(_3\) in MeOH at RT for 6 h. This led to successful conversion of amine \( \text{76} \) to azide \( \text{67} \) in 29\% yield. This method was also employed for the azide transfer of 4-phenylbutan-2-amine \( \text{77} \) in Scheme 32.

![Scheme 32](image)

**Scheme 32**: Azide transfer reaction of 4-phenylbutan-2-amine \( \text{77} \) with Stick reagent \( \text{75} \)\textsuperscript{286}.

This synthesis of azide \( \text{68} \) by diazo transfer of amine \( \text{77} \) with Stick reagent \( \text{75} \) was performed successfully in 29\% yield. The azide transfer of 6-AQ \( \text{78} \) with Stick reagent \( \text{75} \) was also tried in the synthesis of 6-azidoquinoline \( \text{69} \) in Scheme 33.

![Scheme 33](image)

**Scheme 33**: Azide transfer reaction of 6-AQ \( \text{78} \) with Stick reagent \( \text{75} \).
This attempted azide transfer of 6-AQ 78 showed no evidence of the formation of the azide product when monitored by TLC and an alternative method was considered. Previous examples of synthesising azidoquinolines in the literature use either triflic azide or via a route that converts the amine to an intermediate diazonium salt with sodium nitrite before reacting with sodium azide to produce azidoquinoline 287-291. A method performed by Khan et al (1982) using sodium nitrite and sodium azide in the synthesis of an azidoquinolines is shown in Scheme 34.

$$\begin{align*}
\text{H}_2\text{N} & \quad \text{a)} \quad \text{NaNO}_3 \; 1.5 \, \text{eq}, \; 10\% \; \text{HCl aq}, \; 5^\circ\text{C}, \; 1\, \text{h} \\
\text{78} & \quad \text{b)} \quad \text{NaN}_3 \; 2.1 \, \text{eq}, \; 10\% \; \text{HCl aq}, \; 5^\circ\text{C}, \; 1\, \text{h} \\
\text{N}_3 & \quad 10\% \\
\text{69} & 
\end{align*}$$

Scheme 34: Synthesis of 6-azidoquinoline 69.

The reaction of these conditions in Scheme 3.5 produced 69 in 10% yield. Product 66 was then used in the synthesis of 70 in Scheme 35.

$$\begin{align*}
\text{N}_3 & \quad \text{CH}_3\text{I} \\
\text{Dioxane, reflux, 1h} & \\
\text{69} & \quad \text{45\%} \\
\text{N}_3 & \quad \text{70} 
\end{align*}$$

Scheme 35: Methylation of 6-azidoquinoline 69 with iodomethane to the quaternary amine 70 292.

The synthesis of the quaternary amine in Scheme 35 was performed with a method developed by Ghandi et al (2015) by refluxing with iodomethane in dioxane for 1 h. This method resulted in the successful synthesis of the product 70 in 45% yield 292.

To summarise, the synthesis of the azide conjugations partners that will react with the terminal alkyne of labels 35 and 36 was performed successfully for 67, 68, 69 and 70 with 33 being obtained from commercial sources. Azides 67 and 68 were both synthesised in 29% yield. The azide conversion of 78 was unsuccessful with
Stick reagent 75 so was instead synthesised using an alternate method that employed sodium nitrite and sodium azide producing 69 in 10% yield. The addition of the methyl group to form the charged amine of 69 was performed successfully in 45% yield. These azides could now be used in a conjugation reaction with the terminal alkyne group of the label on the labelled glycan.

3.3.2.2 Investigating CuAAC conditions for the reaction of conjugation partner 33 with 35 labelled glycans

The CuAAC reaction between an alkyne group and an azide group in the literature has been performed in a variety of solvents, with a variety of copper salts, reducing agents, bases and at a variety of temperatures. A wide variety of potential conditions identified from the literature were investigated. The conditions for the CuAAC reaction with labelled glycans were trialled to find a set of conditions that produce a fast and high yielding reaction. The conditions investigated in the reaction of glycan label 35 with benzyl azide 33 are shown in Table 5.
## Table 5: Condition of CuAAc reactions attempted.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Solvent A</th>
<th>Solvent B</th>
<th>Ratio of A/B</th>
<th>Copper salt</th>
<th>Reducing agent</th>
<th>Base</th>
<th>Temperature (°C)</th>
<th>Ref</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>DMF</td>
<td>None</td>
<td>N/A</td>
<td>Copper sulfate 0.2 eq</td>
<td>Sodium ascorbate 0.4 eq</td>
<td>None</td>
<td>60</td>
<td>293</td>
</tr>
<tr>
<td>2</td>
<td>MeCN</td>
<td>H$_2$O</td>
<td>2:1</td>
<td>Copper sulfate 0.3 eq</td>
<td>sodium ascorbate 0.3 eq</td>
<td>None</td>
<td>RT</td>
<td>294</td>
</tr>
<tr>
<td>3</td>
<td>DMSO</td>
<td>None</td>
<td>N/A</td>
<td>Copper bromide 0.1 eq</td>
<td>N/A</td>
<td>NEt$_3$</td>
<td>0.4 eq</td>
<td>RT</td>
</tr>
<tr>
<td>4</td>
<td>MeOH</td>
<td>None</td>
<td>N/A</td>
<td>Copper acetate 0.1 eq</td>
<td>Sodium ascorbate 0.1 eq</td>
<td>None</td>
<td>100</td>
<td>296</td>
</tr>
<tr>
<td>5</td>
<td>DMSO</td>
<td>H$_2$O</td>
<td>9:1</td>
<td>Copper sulfate 0.1 eq</td>
<td>Sodium ascorbate 0.2 eq</td>
<td>Potassium carbonate 1.2 eq</td>
<td>65</td>
<td>297</td>
</tr>
<tr>
<td>6</td>
<td>H$_2$O</td>
<td>None</td>
<td>N/A</td>
<td>Copper sulfate 0.05 eq</td>
<td>Sodium ascorbate 0.15 eq</td>
<td>None</td>
<td>RT</td>
<td>298</td>
</tr>
<tr>
<td>7</td>
<td>DCM</td>
<td>None</td>
<td>N/A</td>
<td>Copper bromide 0.02 eq</td>
<td>N/A</td>
<td>DIPEA 0.04 eq</td>
<td>RT</td>
<td>299</td>
</tr>
<tr>
<td></td>
<td>Solvent</td>
<td>Additives</td>
<td>Ratio</td>
<td>Copper compound</td>
<td>Other additives</td>
<td>Temperature</td>
<td>Reaction Time</td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---------</td>
<td>-----------</td>
<td>-------</td>
<td>-----------------</td>
<td>----------------</td>
<td>-------------</td>
<td>--------------</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Dioxane</td>
<td>None</td>
<td>N/A</td>
<td>Copper bromide</td>
<td>N/A</td>
<td>N/A</td>
<td>RT</td>
<td>300</td>
</tr>
<tr>
<td>9</td>
<td>MeCN</td>
<td>H₂O</td>
<td>10:1</td>
<td>Copper sulfate</td>
<td>Sodium ascorbate</td>
<td>0.1 eq</td>
<td>1.1 eq</td>
<td>RT</td>
</tr>
<tr>
<td>10</td>
<td>MeOH</td>
<td>H₂O</td>
<td>1:1</td>
<td>Copper sulfate</td>
<td>Sodium ascorbate</td>
<td>0.07 eq</td>
<td>0.14 eq</td>
<td>None</td>
</tr>
<tr>
<td>11</td>
<td>H₂O</td>
<td>BuOH</td>
<td>2:1</td>
<td>Copper sulfate</td>
<td>Sodium ascorbate</td>
<td>0.01 eq</td>
<td>0.1 eq</td>
<td>None</td>
</tr>
<tr>
<td>12</td>
<td>EtOH</td>
<td>None</td>
<td>N/A</td>
<td>Copper bromide</td>
<td>N/A</td>
<td>0.05 eq</td>
<td>None</td>
<td>RT</td>
</tr>
<tr>
<td>13</td>
<td>H₂O</td>
<td>Acetone</td>
<td>1:1</td>
<td>Copper sulfate</td>
<td>Sodium ascorbate</td>
<td>0.1 eq</td>
<td>0.2 eq</td>
<td>None</td>
</tr>
<tr>
<td>14</td>
<td>MeCN</td>
<td>None</td>
<td>N/A</td>
<td>Copper sulfate</td>
<td>Sodium ascorbate</td>
<td>0.05 eq</td>
<td>0.2 eq</td>
<td>None</td>
</tr>
<tr>
<td>15</td>
<td>DMF</td>
<td>None</td>
<td>N/A</td>
<td>Copper bromide</td>
<td>N/A</td>
<td>1 eq</td>
<td>None</td>
<td>RT</td>
</tr>
</tbody>
</table>
These conditions were initially trialled in a triazole forming reaction between label 35 without carbohydrate and an excess of commercially sourced benzyl azide 33 in each of the conditions from Table 5. The reactions were monitored by TLC after 4 h which is shown in Figure 49.

Figure 49: TLC monitoring of the CuAAc between alkyne label 35 without added carbohydrate and benzyl azide 33. The numbers denote the conditions from Table 5. Az is the spot of benzyl azide. Al is the spot of alkyne label 35. SO₄ and Br are the spots for copper sulfate and copper bromide respectively. Asc is the spot of sodium ascorbate.

Figure 49 shows the TLC plate of the conjugation reaction from the conditions in Table 5 visualised by UV at 254 nm. The numbers in the figure denote the conditions used for that spot. The formation of the triazole product was determined by the absence of the UV absorbance corresponding to the alkyne label 35 at retention factor (Rf) 0.2. The absence of this spot shows the disappearance of the alkyne label in solution, a result of the conjugation of the label to the azide. In this case, conditions 2, 6, 12 and 14 showed the desired reduction of alkyne observed through the disappearance of the spot at Rf 0.2. As these conditions were also considered not to result from solubility issues for either the labelled carbohydrate or azide these conditions were taken forward to the next trials. In these trials, the CuAAc reaction was performed with a labelled carbohydrate. In this case, lactose was labelled with 35 and reacted with benzyl azide 33. The reaction conditions were altered with heating at 40 °C for 4 h, rather than the temperatures...
in Table 5. The reactions were monitored by TLC after 4 h and this TLC plate is shown in Figure 50.

![TLC plate with labels](image)

**Figure 50:** TLC monitoring of the CuAAC between lactose that had been labelled with alkyne tag 35 and benzyl azide 33 under conditions 2, 6, 12 and 14 from Table 5. The numbers denote the conditions from Table 3. Az is the spot of benzyl azide 33. Al is the spot of alkyne label 35. SO$_4$ and Br are the spots for copper sulfate and copper bromide respectively. Asc is the spot of sodium ascorbate.

In these reactions, the conditions that displayed the greatest decrease in the spot corresponding to the azide 33 indicated the formation of the triazole product were deemed to be the best. In these trials, conditions 2 and 12 showed the largest decrease in the azide. These reactions were repeated on a larger scale at 40 °C for 4 h, the resulting reaction mixture was purified by SPE with Spe-ed2 amide-SPE (Applied Separations Inc.) cartridges then analysed by HPLC-FLD. The traces produced by these injections are shown in Figure 51.
Figure 51: HILIC-FLD trace of the product of the CuAAC reaction between 33 and 35 labelled lactose performed by a) condition 2 and b) 12.

Figure 51 (a) shows the injection of the CuAAC product of the reaction with condition 2. This trace clearly shows a single peak at 8 mins, likely caused by the triazole product of the reaction between 33 and 35 labelled lactose. No peak is present in Figure 51 (a) that could correspond to the unreacted labelled carbohydrate.
Figure 51 (b) shows the FLD trace of the injection for the sample produced in condition 12. This trace, unlike Figure 51 (a), is distinctly different, Figure 51 (b) containing three large peaks after 8 mins, the first appearing at the same retention as the peak in Figure 51 (a) likely corresponding to the product of the CuAAc reaction, although this could not be confirmed with FLD detection alone. The peak in Figure 51 (a) was more than a six-fold higher in signal intensity compared to Figure 51 (b). The second peak in Figure 51 (b) at 11 mins likely corresponds to unreacted 35 labelled lactose as it elutes at the same time as the labelled GU2 species in Figure 44 (b). The last peak in Figure 51 (b) however is of unknown origin, a possible explanation for this is a by-product of the CuAAc reaction.

As the structure of the analyte producing these peaks could not be determined by FLD alone, the origin of these peaks were confirmed by MALDI-TOF as MALDI was originally planned to be used for the analysis of collected glycans in later work. The samples were spotted with the matrix super-DHB (sDHB), a 9:1 mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid on an AnchorCHIP plate. The MALDI-TOF MS spectra for both samples is shown in Figure 52.
Figure 52: MALDI-TOF trace of the triazole product of benzyl azide 33 and 35 labelled lactose produced by a) condition 2 and b) condition 12.

In both cases, a peak at m/z 705.345 (M+H)^+ was present which corresponded to the triazole product of the CuAAC reaction between 33 and lactose labelled with 35. Adducts of the product were also observed, of these adducts the sodium adduct was the most abundant with the sodium-acetonitrile and copper adducts also present. Figure 52 (b) also showed peaks corresponding to [M+H]^+, [M+Na]^+ with
the most abundant adduct [M+Cu]+. The increased detection sensitivity for the product of the azide conjugation reaction of the labelled carbohydrate compared to the labelled carbohydrate without azide conjugation in both HPLC-FLD and MALDI-TOF for condition 2 meant that they were selected for future CuAAC with labelled glycans collected from HILIC-FLD analysis.

These conditions would then be applied for the CuAAC reaction of glycans labelled with multifunctional labels 35 and 36 with the azides 33, 67, 68, 69 and 70 synthesised in this chapter. The hydrophobic triazole product of these CuAAC reactions are shown in Table 6.

Table 6: Triazole products of CuAAC reaction between multifunctional labels 35 and 36 and azides 33, 67, 68, 69 and 70. R1 represents the carbohydrate portion of the analyte.

<table>
<thead>
<tr>
<th>Alkyne</th>
<th>Azide</th>
<th>Triazole product</th>
</tr>
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<tbody>
<tr>
<td>35</td>
<td>33</td>
<td>![Image]</td>
</tr>
<tr>
<td>35</td>
<td>67</td>
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<tr>
<td>35</td>
<td>68</td>
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<td>35</td>
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</table>
3.3.2.3 Optimisation of DI-MS analysis of collected 21 and 35 labelled GHP, RNase B and IgG glycans

Once the set of conditions that resulted in the successful formation of the triazole product were identified, the use of this reaction to improve the detection of collected analytes in MS could be investigated. To investigate this, GHP, RNase B glycans and IgG glycans labelled with control label ProA 21 were collected from HILIC-FLD analysis. The ProA 21 labelled samples were analysed to establish a baseline for sensitivity in this analysis. This analysis would act as a comparison to
the analysis of the 35 and 36 labelled carbohydrates after azide conjugation reaction to determine whether azide addition results in a higher detection intensity. Initially, the intention was to analyse these collected samples by MALDI-TOF-MS, however poor detection of the analyte was encountered. An equivalent of 20 HILIC injections were collected for analysis by AnchorChip MALDI with sDHB but no analyte was detected at this concentration (not shown). It is likely therefore that the concentration of these analyte were below the LLOD of the instrument.

For these reasons, ESI-MS was used as an alternative. Once again, 20 injections of the ProA 21 labelled GHP, RNase B glycans and IgG glycans were collected by a fraction collector and dried. The DI-MS analysis of the collected labelled GHP samples was performed with the analyte reconstituted in either solution A, (a 1:1 solution composed of 0.1% FA (aq) and 99.9 % MeCN with 0.1% FA), or solution B, (a 1:1 solution of 0.1% FA (aq) and 99.9 % MeOH 0.1% FA). These systems were used to investigate which would result in the highest detector response of ProA 21 labelled samples. These DI-MS spectra are shown in Figure 53.
Reconstituting the analyte in a mixture of a polar and organic solvents combines the positive droplet properties inherent to organic solvents and the beneficial solubility of polar solvents. Glycan are polar molecules and suffer from poor solubility in less polar solvents such as MeOH and MeCN. Reconstituting labelled glycans in MeOH may mean less of the sample is dissolved in the injection solution resulting in less analyte entering the detector. Polar solvents such as water result in a lower ionisation efficiency. Water has a low vapour pressure and higher surface tension, this means droplets are less volatile and evaporate less leading to larger droplets reduced coulomb fission and reduced ionisation efficiency. Protic additives such as acetic acid and formic acid (FA) at low levels (typically 0.1-0.5%) may also be employed as they facilitate ionisation by donating protons.
The analysis of ProA 21 labelled GU5 in DI-ESI-MS shows clear detection of the analyte at this concentration. The peak at m/z 1048.45 corresponds to the [M+H]^+ species was detected in both Figure 53 (a) and Figure 53 (a). A 9-fold increase in relative abundance was observed for the [M+H]^+ species in Figure 53 (a) compared to this same peak in Figure 53 (b).

Figure 54: DI-MS analysis of ProA 21 labelled GU10 collected from analysis of GHP reconstituted in a) solution B and b) solution A.

This analysis was repeated with collected ProA 21 labelled GU10, a larger GU analyte from the analysis of ProA 21 labelled GHP. This MS spectra of these injections is shown in Figure 54. Figure 54 show the clear detection of the analyte as a mixture of doubly charged [M+H]^2+, [M+H+Na]^2+ and [M+2Na]^2+ species. The
injection of analyte reconstituted in solution A in Figure 54 (a) showed the presence of \([\text{M+2H}]^{2+}\), \([\text{M+H+Na}]^{2+}\) and \([\text{M+2Na}]^{2+}\) at m/z 929.86, m/z 940.85 and m/z 951.84. A further small peak at m/z 938.38 corresponding to the \([\text{M+H+NH}_4]^{2+}\) species was also detected. In contrast, only the \([\text{M+H+Na}]^{2+}\) adduct could be seen for the sample reconstituted solution B in Figure 54 (b) at signals slightly above the baseline.
The next DI-ESI-MS analysis was performed with ProA 21 labelled GU15 in Figure 55. In Figure 55 (a) and Figure 55 (b) the doubly and triply charged adducts of the labelled GU15 analyte were detected for the sample reconstituted in solution A. These doubly charged species were the [M+2H]^2+, [M+H+Na]^2+ and [M+2Na]^2+ adducts at m/z 1343.51, m/z 1345.98 and m/z 1356.98. The triply charged species appeared as [M+H+2Na]^2+ and [M+3Na]^3+ at m/z 904.99 and m/z 912.65. For the MS of the sample dissolved in solution A in Figure 55 (c), no peak corresponding to any triply or doubly charged adducts of ProA 21 labelled GU15 is clearly detected.

The samples reconstituted in solution B clearly and consistently resulted in higher ionisation efficiency. This may be due to MeOH being a protic solvent; protic solvents donate protons resulting in higher ionisation than the samples reconstituted in solution A. Due to this the solution A diluent was chosen for future injections of collected native glycans.

Figure 56 shows the DI-ESI-MS analysis of ProA 21 labelled Man5, Man6 and Man8 glycans collected from an analysis of RNase B glycans. In Figure 56 (a) and Figure 56 (b), analytes are observed as doubly charged [M+2H]^2+, [M+H+Na]^2+ and [M+2Na]^2+ species, these adducts were detected at m/z 727.81, m/z 738.80 and m/z 749.79 for the ProA 21 labelled Man5 species and at m/z 808.4, 819.83, 830.82 for the
ProA 21 labelled Man6 analyte. In Figure 56 (c) only the [M+2H]\(^{2+}\) and [M+H+Na]\(^{2+}\) adducts corresponding to the Man8 labelled species could be detected at m/z 970.89 and m/z 981.88. The most abundant signal in each of the trace in Figure 56 for the labelled Man5, Man6 and Man8 glycans corresponded to the [M+H+Na]\(^{2+}\) adduct.
Figure 56: DI-MS analysis of ProA labelled a) Man5, b) Man6 and c) Man8 glycans collected from analysis of RNase B glycans.

The MS analysis of the equivalent of 20 injection ProA labelled IgG glycans was then performed to confirm whether detection was possible at this concentration. For this, the four most abundant glycans in the IgG glycome, the G0F, G1F, G2F and A1F glycans were collected from the HPLC-FLD analysis and reconstituted in solution B. The DI-ESI-MS analyses of these samples is shown in Figure 57.
Both the G0F and G1F species in Figure 57 (a) and Figure 57 (b) were detected as the doubly charged [M+2H]$^{2+}$, [M+H+Na]$^{2+}$ and [M+2Na]$^{2+}$ species at m/z 841.87, m/z 852.86 and m/z 863.85 for the G0F species and m/z 922.89, 933.88 and 944.87 for the G1F species. The most abundant peak in both spectra was caused by the [M+H+Na]$^{2+}$ species.

The analysis of G2F in Figure 57 (c) and Figure 57 (d) showed the formation of doubly and triply charged species. The triply charged species of G2F were observed as [M+H+2Na]$^{3+}$ and [M+3Na]$^{3+}$ species at m/z 684.27 and m/z 691.60 with doubly charged species present as [M+2H]$^{2+}$, [M+H+Na]$^{2+}$, [M+2Na]$^{2+}$ species at m/z 1004.42, m/z 1014.91 m/z and m/z 1014.91. The most abundant peak corresponded to the [M+H+Na]$^{2+}$ adduct and [M+H+2Na]$^{2+}$ adduct.

The sialylated A1F in Figure 57 (e) glycan could not be detected, this may be due to the negative charge on Neu5Ac 5 which hampers the formation of a positive ion preventing detection. To see if this was the case, the ProA 21 labelled A1F glycan was analysed by negative mode DI-ESI-MS shown in Figure 58.
Figure 58: DI-negative MS analysis of ProA 21 labelled A1F glycans collected from analysis of IgG glycans.

The negative DI-ESI-MS analysis contained a peak at m/z 1147.46 which may correspond to the [M-2H]^{2-} however this peak was at low intensity and was not well resolved from the noise. From these analyses, it could be confirmed that the detection of these samples at this concentration is suitable in most cases. These analyses resulted in spectra where detection of the product was facile and therefore analysis at this concentration was deemed satisfactory. These samples could then be used as comparisons to the azide conjugated 35 and 36 labelled carbohydrates to investigate the ability for the addition of azide to increase detection sensitivity in ESI-MS. These peaks would also be compared to an internal standard that would be at the same concentration across all analyses giving a better comparison for the relative abundance between the azide conjugated labelled glycan and the control 21 labelled glycan.

3.3.2.4 Production of a 21 labelled maltoheptaose internal standard to investigate the ability for azide conjugation to improve MS detection.

Once the satisfactory detection of glycans at this range was established, the ability of the CuAAc reaction to improve detection could be investigated. Initially this was to be performed on a carbohydrate standard before being applied to collected
labelled native glycans. This glycan standard needed to be of comparable GU to the native glycans; the Man5 glycan is 7 GU and the G0F glycan is 8 GU. Therefore, maltoheptaose (a maltooligosaccharide consisting of 7 GU) was selected as a standard of comparable size.

The maltoheptaose standard was labelled with both ProA 21 and its alkyne terminated derivative 35 in the same labelling and purification method used previously. The HPLC-FLD analysis of these samples are shown in Figure 59.

Figure 59: HILIC-HPLC analysis of maltoheptaose labelled with a) ProA 21 and b) derivative 35.
The injection of these two analytes confirmed the successful labelling of the analyte. Additional peaks were present eluting earlier in the trace indicating the presence of impurities which appear to be caused by labelled maltohexaose and maltopentaose. These GU7 analyte were clearly detected at high intensity and could be collected to evaluate the ability for the azide conjugation reaction to increase the MS detector signal in the future.

3.4 Conclusion and future work

The synthesis of the multifunctional labels 35 and 36 has resulted in the formation in two labels that displayed similar detection sensitivity and resolution in HILIC-HPLC-FLD analysis when compared to ProA 21 labelled samples which indicated that these labels could be adopted in conventional N-linked glycan analysis workflows.

The fluorescent wavelengths for both novel labels were determined experimentally and were used to calculate the LLOD of detection for label 35. This was found to be similar to the parent compound 21, with both labels undetectable at concentrations lower than 10 pmolml⁻¹. The linearity of labels 35 and 36 were determined through the production of a standard calibration curve and demonstrated the linearity of the novel labels in the analytical system was comparable with the parent compound 21 at \( r^2 = 0.9997 \) and 0.9999 respectively.

The HILIC-HPLC analysis of GHP, RNase B glycans and IgG glycans labelled with the novel glycan labels 35 and 36 showed satisfactory detection sensitivity, separation and resolution which were clearly similar to the analysis of the same sample labelled with ProA 21. Plotting elution time versus GU for the labelled GHP samples, high coefficient of determination values were calculated for both novel labels 35 and 36 at 0.9988 and 0.9950 respectively which was comparable to the ProA 21 labelled species at 0.9995.
Following the successful analysis of glycans labelled with multifunctional labels 35 and 36, the successful synthesis of Stick reagent 75 led to the synthesis of azide conjugate partners 67, 68, 69 and 70 which could be used in the reaction with labelled glycans to increase detection sensitivity. In total, fifteen CuAAc reaction conditions for the addition azide 33 with label 35 were trialled to determine which resulted in the fastest reaction. In this case, the reaction performed in condition 2, a solution of MeCN : H₂O (2:1) with 0.3 eq of copper sulfate and sodium ascorbate at 40 °C for 4 h was deemed to be the best. These conditions were then used in the azide addition of labelled lactose which produced triazole product as confirmed by MALDI-TOF-MS.

Once the condition of the azide addition reaction was established, samples of labelled glycans were collected and analysed by DI-ESI-MS to determine at what concentration these glycans could be detected. Samples of ProA 21 labelled carbohydrate was analysed to establish a baseline of MS sensitivity which could be compared to the 35 and 36 labelled carbohydrates after the azide conjugation reactions. The diluent that resulted in the greatest detection sensitivity for these samples was investigated with the samples reconstituted in solution B, a 1:1 solution composed of 0.1% FA (aq) and 99.9 % MeOH with 0.1% FA mixture of resulting in greater detection sensitivity. From this analysis it was also concluded that analysis on 20 HILIC injections was satisfactory.

The successful labelling of the maltoheptaose standard was also performed and confirmed by HILIC-HPLC-FLD. This labelled maltoheptaose was produced as an internal standard to investigate the effect of the azide conjugation reaction to in increasing detection sensitivity in subsequent MS analysis of the azide conjugated labelled carbohydrate. Should this reaction result in an increase in detection sensitivity these azides could then be reacted with labelled native glycans in future work to investigate if this increase in detection sensitivity was also observed.

In conclusion, the synthesis of alkyne labels 35 and 36 has led to the production of glycan labels that display similar fluorescent characteristics in HILIC-FLD results to
currently used glycan labels. These labels could be successfully adopted into current glycan analysis workflows. These labels also provide the ability for further reaction of the labelled glycans post separation. However, the ability for these azides when reacted with these labelled glycans to increase detection sensitivity still needs to be established.
3.5 Experimental

*General experimental*

All chemicals were purchased from commercial sources (Fisher Scientific, Sigma Aldrich, Alfa Aesar, Acros Organics, Fluorochem) and used without further purification. NMR solvents were purchased from Sigma Aldrich, Fisher Scientific or Fluorochem. Experiments were monitored TLC. TLCs were carried out on aluminium backed Merck Millipore TLC silica gel 60 F254 plates. Visualisation of TLC plates was accomplished by UV (λ = 254 nm) and either potassium permanganate or ninhydrin stain.

*Analytical equipment*

Proton NMR (δ_H), carbon NMR (δ_C), ATR-IR spectroscopy, MP and HRMS were collected in identical fashion to Chapter 2. Fluorescence spectroscopy was performed on a Varian Eclipse Fluorescence spectrophotometer.

HPLC was performed on an Agilent 1100 series HPLC system. Detection was performed using an Agilent 1100 series G1321A FLD detector. Fractions were collected with an Agilent 1100 series G1364B preparative fraction collector. The column for the separation was a TSKgel® Amide-80 HR (25 cm × 4.6 mm, 5 μm particle size) with a TSK® guardgel Amide-80 (1.5cm × 3.2 mm, 5 μm particle size) (TOSOH Bioscience). Solvent A was composed of ammonium formate (aq) (50 mM, pH 4.4) and solvent B consisted of MeCN. Solutions were filtered and degassed prior to use.

Enzyme concentration was determined with a NanoDrop Lite spectrophotometer at 280 nm and path length of 10 mm. MALDI-TOF was performed on a Bruker Ultraflex previously calibrated with Bruker Peptide Calibration Standard II. MS was performed on a Thermo LTQ Orbitrap XL by DI
Buffers and media

LB medium: 25 g LB medium powder was dissolved in 1 litre of water, sterilized by autoclaving.

Gel electrophoresis: SDS-PAGE gels were manually cast using the procedure outlined below.

Running gel: 15% polyacrylamide gels was made by combining 40% acrylamide and bis acrylamide solution (3 mL), H₂O (2.8 mL), 1.5M Tris pH 8.8 (2 mL), 10% SDS (80 µL), ammonium persulfate (APS) (80 µL) and tetramethylethylenediamine (TEMED) (8 µL). This solution was added to an empty cassette until 2 cm from the top where an overlay of isopropanol was added while the gel was left to solidify before the isopropanol was removed.

Stacking gel: 4% stacking gel was made by combining 40% acrylamide and bis acrylamide solution (0.75 mL), water (2.9 mL), 0.5M Tris pH 6.8 (1.25 mL), 10% SDS (50 µL), APS (50 µL) and TEMED (5 µL). This solution was added onto dried running gel and a well comb was added until gel had solidified. The gel was stored in damp conditions at 4 °C and use within three months of casting.

10 x Reservoir buffer: 30.3 g Tris-HCl, 144 g glycine, 10 g 1% SDS were combined in 1L of dH₂O. Reservoir buffer was diluted 10-fold in H₂O prior to use.

SDS-PAGE gel stain: 500 mL MeOH, 400 mL distilled water, 100 mL glacial acetic acid, 2.5 g Coomassie Brilliant Blue R-250.

SDS-PAGE gel destain: 400 mL MeOH, 530 mL distilled water, 70 mL acetic acid.

2M Ammonium formate pH 4.4 buffer: 184.12 g FA was combined with 1 L water at -10 °C. 25% ammonia solution (200 mL) was added a quarter at a time to prevent rapid temperature rise. 25% ammonia solution (5 mL) was added until pH was 4.4. Solution
was then made to 2 L in water in a volumetric flask. Ammonium formate buffer was diluted 40-fold prior to use.

*Acetate – borate MeOH solution:* 0.6 g sodium acetate trihydrate and 0.3 g boric acid were combined in MeOH (15 mL MeOH).

*Glycan label solution:* Glycan label solutions were prepared at a final concentration of 162.8 mM in MeOH.

*Sodium cyanoborohydride solution:* Sodium cyanoborohydride solution was prepared at 418.8 mM in acetate - borate MeOH solution.

3.5.1 HILIC-HPLC-FLD analysis of 21, 35 and 36 labelled carbohydrates

3.5.1.1 General labelling of a maltooligosaccharide

Glycan label solution (25 µL) and sodium cyanoborohydride solution (15 µL) were added to an 50 µM aqueous maltooligosaccharide solution (10 µL) and heated at 65 °C for 3 h in a water bath. The samples were made up to volume of 1 mL with the addition of MeCN. A white precipitate formed. Derivatized carbohydrates were purified by SPE with Spe-ed2 amide-SPE (Applied Separations Inc.) cartridges (1 mL, 25 mg bed volume) which were pre-equilibrated with MeOH (2 x 1 mL). The reaction solution was loaded onto the SPE and allowed to drip through the column. The cartridge was washed with MeCN : H₂O (5 x 1 mL, 99:1, v/v) followed by MeCN : H₂O (5 x 1 mL, 97:3, v/v). The labelled oligosaccharides were eluted with water (0.6 mL) and dried using a vacuum centrifuge concentrator. Dried sample was stored at -20 °C.

3.5.1.2 3D fluorescence spectroscopy of labelled maltopentaose

Labelled maltopentaose (40 nmol) was dissolved in water (500 µL) and added to a fused quartz fluorescent cuvette. The 3D fluorometry of labelled carbohydrate was
performed between 250 and 500 nm, increasing the excitation wavelength by 10 nm in each subsequent scan. Emission was recorded between 300 nm and 600 nm, with a slit distance of 5 nm.

3.5.1.3 Labelling of a lactose standard

Glycan label (83.2 µmol) and sodium cyanoborohydride (15.7 mg, 249.6 µmol, 3 eq) was dissolved in acetate-borate MeOH solution (16 mL). Lactose (28.448 mg, 83.2 µmol, 1 eq) was dissolved in water (4 mL) then added dropwise to the labelling solution. This solution was stirred at 65 °C for 24 h. The solution was then dried in vacuo. The dried samples were dissolved in a mixture of MeCN (9.5 mL) and H₂O (0.5 mL) and purified by amide SPE. This solution was loaded onto Spe-ed2 amide (Applied Separations Inc.) SPE cartridge (1 mL, 25 mg bed volume) and allowed to drip through. The sorbent was washed with MeCN : H₂O (10 x 1 mL, 99:1, v/v) followed by MeCN : H₂O (10 x 1 mL, 97:3, v/v). The labelled oligosaccharides were eluted with H₂O (5 x 1 mL). The elution fractions containing carbohydrate were determined by TLC, then combined. The combined solution was dried by vacuum centrifugation and pure solid was dissolved to a solution with a final concentration of 100 µM.

3.5.2 Preparation of carbohydrates

3.5.2.1 Production of a GHP from dextran

Dextran from Leuconostoc mesenteroides (Mr ~200,000, 1 g) was added to 0.1 M HCl (10 mL) and stirred at 100°C for 4 h. Afterwards, the solution was left to cool, Amberlite (2 g) was added and stirred at RT until solution reached pH 7 on pH indicator paper. The neutralised solution was collected, the solvent was adjusted to make final solution 0.1 mg/ml in water.

3.5.2.2 Expression of PNGase F
BL21(DE3) overnight cultures (15 mL) bearing pOPH6 (PNGase F) (a gift from Shaun Lott - addgene plasmid # 40315) were grown at 37 °C with 100 µg/mL ampicillin. 0.8% final culture volume was used to inoculate 1 L of fresh LB media with an identical concentration of antibiotic. This culture was then grown (37 °C, 210 rpm) until OD$_{600}$ reached 0.5-0.7, when it was then induced with IPTG (1 mM final concentration). The culture was left shaking (30 °C, 210 rpm) overnight before it was centrifuged (8000 g, 30 mins, 4 °C).

Expressed recombinant protein was purified using a variation of the methodology described by Loo et al. The cell pellet was re-suspended in 2% culture volume of chilled 0.5 M sucrose, 0.1 M Tris-HCl (pH 8) and incubated for 20 mins at 4 °C. The lysate was pelleted (8000 g, 20 mins, 4 °C) and re-suspended in 2% original culture volume of chilled ddH$_2$O and incubated at 4 °C for 20 mins with shaking. MgCl$_2$ was added to a final concentration of 20 mM and lysate incubated for a further 20 min with shaking. The sample was centrifuged (8000 g, 20 mins, 4 °C). The supernatant was pipetted off, supernatant was added to a binding buffer composed of an aqueous solution of HEPES (20 mM), NaCl (300 mM) and imidazole (20 mM). This solution was loaded onto a HisTrap HP nickel affinity column (5 mL, GE Healthcare) previously equilibrated with 5 column volumes (CV) of distilled water followed by 5 CV of binding buffer.

The protein solution was absorbed onto the column, and the column then washed with 10 CV of binding buffer. Following the washing step, remaining protein was eluted with elution buffer an aqueous solution composed of HEPES (20 mM), NaCl (300 mM) and imidazole (300 mM). Fractions were analysed for target protein by SDS-Page (12%), the gels were run at 150 V until the marker was level with the base of the running gel. The gels were removed from the cassettes and stained with Coomassie stain. Following gel destaining with gel destain solution, fractions containing target protein were pooled and solution was dialysed O/N at 4 °C in 2L of aqueous dialysis buffer (Tris-HCl (20 mM), NaCl (50 mM), Na$_2$EDTA (5 mM), pH 7.5 at 25°C). The protein sample was concentrated by centrifugation at 4000 g in a centrifugal filter a with molecular weight cut off (MWCO) of 15 kDa. The final
concentration of protein was confirmed by nanodrop and protein solution was stored at a concentration of 1.091 mgmL\(^{-1}\) at 4 °C.

3.5.2.3 Evaluating the activity of recombinant PNGase F

The activity of purified recombinant PNGase F was tested against the deglycosylation of RNase B. RNase B (100 µg) was added to denaturing buffer (30 µL, 0.5% SDS in 40 mM DTT) to a final volume of 300 µL in PBS and heated at 95 °C for 10 mins. The mixture was cooled on ice and 100 µg recombinant PNGase F was added with phosphate buffered saline to a final volume of 600 µL. The sample was incubated O/N, while shaking at 37 °C.

The solution was evaporated to dryness in a vacuum concentrator. Samples were dissolved in H\(_2\)O (500 µL) and centrifuged in a bench top centrifuge at 9,000 g for 10 mins. Supernatant was collected and loaded onto a PGC SPE cartridge that had previously been equilibrated and primed. SPE was equilibrated with MeOH (1 mL), sodium hydroxide 1N (1 mL), H\(_2\)O (1 mL), acetic acid 30% (1 mL) and then finally H\(_2\)O (1 mL), then primed with buffer B composed of an aqueous solution of 50% MeCN and 0.1% TFA (1 mL), then washed with buffer A composed of an aqueous solution of 5% MeCN and 0.1% TFA (1 mL). The supernatant was then loaded and allowed to drip through, washed with H\(_2\)O (1 mL), then buffer A (1 mL) and eluted with buffer B (1 mL).

The activity of purified recombinant PNGase F was tested against RNase B. RNase B (20 µg) was added to denaturing buffer (1 µL, 0.5% SDS in 40 mM DTT) to a final volume of 10 µL in PBS and heated at 95 °C for 10 mins. The mixture was cooled on ice and 5 µg recombinant PNGase F was added with PBS to a final volume of 20 µL. The sample was incubated at 37 °C while shaking for 90 minutes. Digestion was stopped with the addition of 4 µL 4X LDS loading dye and samples were analysed on a 15 % denaturing SDS-PAGE gel and visualised with Coomassie stain (Figure 60).
3.5.2.4 Deglycosylation of RNase B

RNase B (1 mg) was dissolved in H$_2$O (270 µL). To this, denaturing solution (0.5% SDS, 40mM dithiothreitol, 30 µL) was added. This solution was shaken at 95 °C for 10 mins. After this time the tube was cooled to RT on ice. PBS (300 µL) and PNGase F (100 µg) was added and the tube was shaken at 37 °C overnight.

The solution was then evaporated to dryness in a vacuum concentrator. Samples were dissolved in H$_2$O (500 µL) and centrifuged in a bench top centrifuge at 9,000 g for 10 mins. Supernatant was collected and loaded on a Hypercarb Hypersep PGC SPE (Thermo Scientific) cartridge that had been equilibrated and primed. SPE was equilibrated with MeOH (1 mL), sodium hydroxide 1N (1 mL), H$_2$O (1 mL), acetic acid 30% (1 mL) and then finally H$_2$O (1 mL). SPE was then primed with buffer B composed of an aqueous solution of 50% MeCN and 0.1% TFA (1 mL), then washed with buffer A, composed of an aqueous solution of 5% MeCN and 0.1% TFA (1 mL).

The applied glycans were washed with H$_2$O (1 mL), then buffer A (1 mL) then finally, glycans were eluted with buffer B (1 mL). The solution was evaporated first by flowing
nitrogen gas over the solution to remove MeCN then in vacuo in a vacuum concentrator. Dried samples were dissolved in H$_2$O (100 µL) and stored at -20 °C.

3.5.2.5 Deglycosylation of IgG

Human IgG (100 µg) was dissolved in H$_2$O (270 µL). To this a denaturing solution (0.5% SDS, 40mM dithiothreitol, 30 µL) was added. This solution was shaken at 95 °C for 10 mins. After this time the tube was cooled to RT in ice. PBS (300 µL) was added and PNGase F (100 µg) was added and the tube was shaken at 37 °C overnight.

The solution was then evaporated to dryness in a vacuum concentrator. Samples were dissolved in H$_2$O (500 µL) and centrifuged in a bench top centrifuge at 9,000 g for 10 mins. Supernatant was collected and loaded on Hypercarb Hypersep PGC SPE (Thermo Scientific) cartridge that had been equilibrated and primed. SPE was equilibrated with MeOH (1 mL), sodium hydroxide 1N (1 mL), H$_2$O (1 mL), acetic acid 30% (1 mL) and then finally H$_2$O (1 mL), then primed with buffer B composed of an aqueous solution of 50% MeCN and 0.1% TFA (1 mL) then washed with buffer A composed of an aqueous solution of 5% MeCN and 0.1% TFA (1 mL).

The applied glycans were washed with H$_2$O (1 mL), then buffer A (1 mL), then finally glycans were eluted with buffer B (1 mL). The solution was evaporated first by flowing gas over the solution to remove MeCN then in vacuo in a vacuum concentrator. Dried samples were dissolved in H$_2$O (100 µL) and stored at -20 °C.

3.5.3 Labelling and purification of labelled carbohydrates for HILIC-HPLC-FLD

3.5.3.1 General labelling of a GHP

Glycan label solution (25 µL) was combined with sodium cyanoborohydride solution (15 µL). Hydrolysed dextran solution (10 µL, 0.1 mgmL$^{-1}$) was added to the labelling solution. The solution was vortexed and then heated at 65 °C for 3 h in a water bath.
Labelled oligosaccharides were purified by SPE with Spe-ed2 amide-SPE (Applied Separations Inc.) cartridges (1 mL, 25 mg bed volume).

Cartridges were pre-equilibrated with MeOH (2 x 1 mL). The carbohydrate labelling solutions were made up to 1 ml with the addition of MeCN. Often a white precipitate formed. This solution was loaded onto the SPE and allowed to drip through the cartridge. The cartridge was washed with MeCN (aq) (5 x 1 mL, 99:1, v/v) followed by MeCN (aq) (5 x 1 mL, 97:3, v/v). The labelled oligosaccharides were eluted with H₂O (0.6 mL) and dried with a vacuum centrifuge concentrator. Dried sample was stored at -20 °C. Samples were reconstituted in H₂O (20 µL) and an injection volume of 1 µL was injected for HPLC analysis.

3.5.3.2 Labelling of glycans released from natural sources

Label solution (25 µL) was combined with a sodium cyanoborohydride solution (15 µL). Dried enzymatically released glycans dissolved in H₂O (10 µL) were combined with the first solution and the mixture was heated at 65 °C for 3 h in a water bath. Derivatized carbohydrates were purified through by SPE with Spe-ed2 amide-SPE (Applied Separations Inc.) cartridges (1 mL, 25 mg bed volume).

Cartridges were pre-equilibrated with MeOH (2 x 1 mL). The carbohydrate labelling solutions were made up to 1 ml with the addition of MeCN. Often a white precipitate formed. This solution was loaded onto the SPE and allowed to drip through the cartridge. The cartridge was washed with MeCN/H₂O (5 x 1 mL, 99:1, v/v) followed by MeCN/H₂O (5 x 1 mL, 97:3, v/v). The labelled oligosaccharides were eluted with H₂O (0.6 mL) and dried with a vacuum centrifuge concentrator. Dried sample was stored at -20 °C. Samples were reconstituted in H₂O (20 µL) and an injection volume of 1 µL was injected in HPLC analysis.

3.5.4 HILIC-HPLC-FLD analysis of labelled carbohydrates
The separation of carbohydrates was performed on a TSKgel® Amide-80 HR (25 cm × 4.6 mm, 5 μm particle size) with a TSK® guardgel Amide-80 (1.5 cm × 3.2 mm, 5 μm particle size) (TOSOH Bioscience). Solvent A was composed of ammonium formate (aq) (50 mM, pH 4.4) and solvent B consisted of MeCN. The flow rate was set at 0.7 mLmin⁻¹ and oven temperature was set to 20 °C. The analysis gradient started at 65% of solvent A which was decreased to 50% over 40 mins. Washing and reequilibration was performed by increasing solvent A to 100% over the next 5 mins which was kept for a further 5 mins then brought back to 65% in 5 mins which was maintained for a further 5 mins.

3.5.5 Synthesis of an azide library

**Imidazole-1-sulfonyl azide 75**

![Imidazole-1-sulfonyl azide 75](image)

Sodium azide (5.0 g, 77 mmol, 1 eq) was added into a round bottom flask with a nitrogen environment. To this, dry ethyl acetate (77 mL) was added, and the resulting suspension was cooled to 0 °C. Sulfuryl chloride (6.2 mL, 77 mmol, 1 eq) was added dropwise over 20 mins with stirring whilst the inert nitrogen atmosphere was maintained. The mixture was then allowed to warm to RT and was stirred for at least 17 h. The temperature of the suspension was again reduced to 0 °C and imidazole (10.0 g, 147 mmol, 1.91 eq) was added over 10 mins whilst nitrogen atmosphere was maintained. The thick suspension was stirred for at least 3 h at 0 °C. A solution of aqueous saturated sodium hydrogen carbonate (150 mL) was then added to basify the reaction mixture. The organic layer was washed with H₂O (150 mL) and dried over magnesium sulfate.

The solution was filtered and cooled to 0 °C and placed under a nitrogen atmosphere. Concentrated sulphuric acid (4.1 mL, 77 mmol, 1 eq) was added dropwise over the
course of 5 mins. The temperature of the mixture was allowed to rise to RT with vigorous stirring over 30 mins. A precipitate had formed which was filtered and washed with a minimum of cold ethyl acetate, yielding white crystals. These crystals were left to dry for 15 mins then collected and dried further under reduced pressure to afford imidazole-1-sulfonyl azide. The reagent was stored under a nitrogen atmosphere at 4°C. (10.38 g, 60 mmol, 78%).

**Rf** DCM : MeOH, 9 : 1, 0.9. **MP** 101-103 °C. **1H NMR** (400 MHz, D2O) δH 7.59 (dd, J = 1.2, 0.7 Hz, 1H, N-CH-CH) 7.98 (t, J = 1.9 Hz, 1H, N-CH-CH), 9.48 (t, J = 1.2 Hz, 1H, N-CH-N). **13C NMR** (101 MHz, D2O) δC 120.24 (N-CH-CH), 122.14 (N-CH-CH), 137.55 (N-CH-N). (As previously noted, decomposition occurred in D2O evidence by additional peak in both **1H NMR** δH (8.57 (s), 7.35 (d, J = 0.6 Hz) and **13C NMR** δC (133.31, 118.83).

**IR** (ATR-FTIR) IR cm⁻¹: 1098 (s, S=O symmetric stretch), 1302 (m, S=O asymmetric stretch), 1429 (m, C=C bend aromatic), 2178 (m, azide stretch), 2872 (m, O-H stretch), 3001 (m, C-H aromatic stretch) 3085 (m, C-H aromatic stretch) 3157 (m, C-H aromatic stretch). **HRMS.** (ESI+) C3H5N3O6S2⁺ expected: 174.0080 (M⁺) found: 174.0078 (M⁺).

(3-azidobutyl)benzene 67

![67](image)

(3-aminopropyl)benzene 76 (200 mg, 1.48 mmol) was added to imidazole-1-sulfonyl azide 75 (457 mg, 1.69 mmol, 1.14 eq) and potassium carbonate (410 mg, 2.96 mmol, 2 eq) in MeOH (8 mL). Aqueous 1% copper sulfate solution (10 µL) was added and the reaction mixture was stirred at RT for 6 h. After 6 h the solvent was removed in vacuo, and a minimum of ethyl acetate was added to dissolve crude product. The solvent was filtered to remove metal salts and ethyl acetate was removed in vacuo to leave crude azide as a colourless liquid. The product was purified by flash chromatography (ethyl acetate : petroleum ether, 1: 1) providing the pure azide as a colourless liquid. (68 mg, 0.42 mmol, 28.5%)
Rf DCM, 0.9. $^1$H NMR (400 Mhz, MeOH-d4) δ_H 1.86 – 2.02 (m, 2H CH₂CH₂CH₂), 2.72 (t, J = 7.6 Hz, 2H, N₃CH₃), 3.30 (t, J = 6.8 Hz, 2H ), 7.18 – 7.25 (m, J = 11.9, 7.4 Hz, 3H, overlapping aromatic CH), 7.28 – 7.37 (m, J = 7.4 Hz, 2H, aromatic CH). $^{13}$C NMR δ_c 30.44 (CH₂CH₂CH₂), 32.77 (CCH₂CH₂), 50.65 (N₃CH₂), 126.15 (aromatic C), 128.46 (2 x aromatic C), 128.52 (2 x aromatic C), 140.85 (aromatic C). IR (ATR-FTIR) IR cm⁻¹ 1453 (m, C-H alkane bend) 2100 (s, azide stretch) 2863 (w, C-H stretch alkane) 2936 (w, C-H stretch alkane) 3028 (w, C-H stretch aromatic) 3368 (w, C-H stretch aromatic).

(3-azidobutyl)benzene 68

(3-aminobutyl)benzene 77 (200 mg, 1.34 mmol) was added to imidazole-1-sulfonyl azide 75 (436 mg, 1.61 mmol, 1.2 eq) and potassium carbonate (371 mg, 2.68 mmol, 2 eq) in MeOH (8 mL). Aqueous 1% copper sulfate solution (10 µL) was added and the reaction mixture was stirred at RT for 6 h. After 6 h, the solvent was removed in vacuo, and a minimum of ethyl acetate was added to dissolve crude product. The solvent was filtered to remove metal salts and ethyl acetate was removed in vacuo to leave crude azide as a colourless liquid. The product was purified by flash chromatography (ethyl acetate : petroleum ether, 1: 1) providing the pure azide as a colourless liquid. (68 mg, 0.39 mmol 29%)

Rf DCM, 0.9. $^1$H NMR δ_H 1.30 (d, J = 6.5 Hz, 3H, CHCH₃), 1.68 – 1.90 (m, 1H, CH₃CH), 2.59 – 2.82 (m, 2H, CH₂CH), 3.52 – 3.66 (m, 2H, CH₂CH₂), 7.24 – 7.17 (m, J = 7.6 Hz, 3H, overlapping aromatic CH), 7.30 (t, J = 7.4 Hz, 2H, aromatic CH). $^{13}$C NMR δ_c 19.53 (CH₂CH₃), 32.38 (CH₂, 37.95, 57.21 (CHN₃), 126.10 (aromatic CH), 128.47 (2 x aromatic CH), 128.54 (2 x aromatic CH), 141.28 (aromatic CH). IR (ATR-FTIR) IR cm⁻¹ 2095 (s,
azide stretch) 2862 (w, C-H stretch alkane) 2930 (w, C-H stretch alkane) 2971 (w, C-H stretch alkane) 3028 (w, CH stretch aromatic).

6-azidoquinoline 69

![6-azidoquinoline 69](image)

Sodium nitrite (103 mg, 1.5 mmol, 1.5 eq) was dissolved in a solution of 10% HCl (aq) (0.6 mL) cooled to 0 °C. 6-AQ 78 (144 mg, 1 mmol) was always dissolved in 10% HCl (aq) (0.6 mL) and added dropwise to the sodium nitrite solution whilst keeping the temperature below 5 °C. This solution was stirred at below 5 °C for 1 hour before addition of sodium azide (140 mg, 2.1 mmol, 2.1 eq) dissolved in 10% HCl (aq) (0.6 mL), which was added dropwise whilst the temperature remained below 5 °C. Effervescence was observed. The flask was stirred for 1 h whilst the temperature was allowed to rise to RT.

After 1 h, the solution was made alkaline by the addition of sodium hydrogen carbonate (5 mL), the product was extracted with DCM (3 x 5 mL). The organic solvent was dried with magnesium sulfate, the solution was filtered and dried in vacuo to afford 6-azidoquinoline 69 as an orange oily solid. (17.0 mg, 0.1 mmol, 10%)

**Rf** DCM : MeOH, 49 : 1, 0.7. **MP** 108 – 110 °C. **1H NMR** (400 MHz, MeOD₄) δH 7.46 (dd, J = 9.0, 2.5 Hz, 1H, N₃CCHCH aromatic), 7.53 (dd, J = 8.4, 4.3 Hz, 1H, NCHCH aromatic), 7.60 (d, J = 2.4 Hz, 1H, N₃CCH aromatic), 8.02 (d, J = 9.0 Hz, 1H, NCHCHCH aromatic), 8.30 (d, J = 8.3 Hz, 1H, N₃CCH aromatic), 8.78 (d, J = 4.0 Hz, 1H, NCH aromatic). **13C NMR** (101 MHz, MeOD₄) δc 116.78 (N₃C), 123.41 (N₃CHCH), 124.03 (N’CHCH), 130.64 (N’CC), 131.21 (N’CH), 137.40 (N’CC), 140.28 (N₃C), 146.50 (N’CH), 150.68 (N’CHCHCH). **IR (ATR-FTIR)** IR cm⁻¹ 1106 (s, C-N stretch), 1500 (s, C-C stretch) 1593 (m, C=C stretch) 2113 (s, azide stretch) 3091 (m, b, C-H aromatic stretch) 3235
(s, b, C-H aromatic stretch) 3355 (s, b, C-H aromatic stretch). **HRMS** (ESI+) C6H7N4+ expected: 171.0665 (M+H+) found: 171.0669 (M+H+).

6-azido-1-methylquinolin-1-ium 70

![N3](attachment:image)

6-aziodquinoline 69 (45 mg, 0.26 mmol) was dissolved in dioxane (1 mL). To this solution, iodomethane (160 mg, 1.13 mmol, 4.3 eq) was added and the solution was refluxed at 120 °C for one hour. After this, the solution was cooled and pipetted off. Ethyl acetate (3 x 5 mL) was added to dissolve by products, followed by addition of petroleum ether (40–60) (3 x 5 mL) for the same purpose. The red solution was dried under reduced pressure to yield pure 6-azido-1-methylquinolin-1-ium 70 (22 mg, 0.118 mmol, 45%).

**Rf** DCM : MeOH : AcOH, 9 : 18 : 1, 0.1. **MP** 214 – 217 °C. **1H NMR** (400 MHz, MeOD4) δH 4.70 (s, 3H, N+CH3), 7.95 (dd, J = 9.5, 2.5 Hz, 1H, N3CCH aromatic), 8.07 (dd, J = 8.5, 5.8 Hz, 1H, (N3CHCH aromatic), 8.11 (d, J = 2.5 Hz, 1H, N3CCH aromatic), 8.53 (d, J = 9.4 Hz, 1H, N3CCH aromatic), 9.12 (d, J = 8.5 Hz, 1H, N3CH aromatic), 9.29 (d, J = 5.7 Hz, 1H, N3CHCHCH aromatic). **13C NMR** (101 MHz, MeOD4) δC 46.46 (N3CH3), 117.07 (N3CCH), 120.40 (N3CHCH), 122.47 (N3CHCH), 128.29 (N3CC), 128.96 (N3CH), 138.81 (N3CC), 144.86 (N3C), 145.92 (N3CH), 148.58 (N3CHCHCH). **IR** (ATR-FTIR) IR cm⁻¹ 1522 (s, C-C stretch), 1621 (m, C=C stretch), 2114 (s, azide stretch), 2923 (m, b, C-H aromatic stretch), 3024 (m, b, C-H aromatic stretch), 3225 (s, b, C-H aromatic stretch), 3369 (s, b, C-H aromatic stretch). **HRMS** (ESI+) C10H10N4+ expected: 185.0822 (M+) found: 185.0821 (M+).

3.5.6 General click reaction of alkyne labelled carbohydrates
Azide (0.588 µmol) was dissolved in MeCN (20 µL). Dried collected carbohydrate was dissolved in MeCN (20 µL). Aqueous copper sulfate solution (176.41 µM, 10 µL, 0.3 eq) and aqueous sodium ascorbate solution (176.41 µM, 10 µL, 0.3 eq) were both added to the azide solution. This solution was heated at 40 °C for 4 h whilst shaking.

After 4 h, the solution was made up to 1 mL with MeCN and added to SPE with Speed2 amide-SPE (Applied Separations Inc.) cartridges (1 mL, 25 mg bed volume) that had been equilibrated with MeOH (1 mL). The carbohydrate solution was allowed to drip through and samples were washed with MeCN/H₂O (3 x 1 mL, 99:1, v/v) followed by MeCN/H₂O (3 x 1 mL, 97:3, v/v). Purified clicked glycans were eluted with water (0.6 mL) and dried in a vacuum centrifuge. Dried samples were dried at -20 °C.

3.5.7 MALDI-TOF analysis of labelled carbohydrates

MALDI-MS was calibrated with Bruker peptide calibration standard II dissolved in TA30 (MeCN:0.1% TFA (aq) (30:70 (v/v)) with HCCA matrix in TA30 to a final concentration of 0.7 mgmL⁻¹. Calibration standard (1 µL) was spotted onto an 800 µm spot anchor chip plate and left to air dry. Matrix solution (0.5 µL) was then spotted and left to dry.

Dried derivatized carbohydrate samples were dissolved in TA30 (2 µL), sDHB was dissolved in a solution of MeCN: H₂O (85:15) and 0.1% TFA to a final concentration of 10 mgml⁻¹. The solution of matrix (0.5 µL) was immediately spotted on an 800 µm spot on an anchor chip plate and allowed to air dry. At total dryness, carbohydrate solution (1 µL) was spotted and allowed to air dry. Samples were analysed in reflectron positive mode between 500 and 4000 m/z with a laser power of 30% and reflector value of 30. 3000 shots were performed for each sample.

3.5.8 DI MS of collected labelled carbohydrates
Collected glycans were dried in a centrifugal concentrator. Dried glycans were reconstituted in H₂O : MeOH (1:1) and 0.1% FA (100 µL). Samples were analysed by MS by DI-MS on a Thermo LTQ Orbitrap XL in either positive or negative ion mode. The analysis was averaged over 20 scans at 100,000 resolution.
Chapter 4 - Reversed phase high performance liquid chromatography analysis of labelled glycans

4.1 Background

HILIC is a common way of analysing polar and charged molecules\(^{310}\). HILIC is widely employed in the analysis of nucleotides, amino acids, peptides, hydrophilic drugs, and oligosaccharides\(^{195, 311-314}\). HILIC is sometimes wrongly considered as a form of NP-HPLC, which is understandable as they both employ polar stationary phases. Typical polar stationary phases consist of bare silica or silica gels modified with polar functional groups such as hydroxyl, cyano, amino and amide groups\(^{195}\). Though both modes use polar stationary phases, the mechanism behind the separations are different. The mechanism of retention in NP-HPLC is due to the adsorption of analyte to the stationary phase whereas HILIC is a form of liquid-liquid partition chromatography\(^{315}\). This is where retention occurs due to interactions between two liquid phases. The partitioning of the mobile phase in the column produces an immobilised layer of water on the stationary phase. The presence of water in the mobile phase is essential for separation. A water component of at least 2-3\% in the mobile phase is required to produce this layer\(^{195, 315, 316}\). The low percentage of water in the mobile phase at the beginning of the run produces a tight layer of water approximately 4Å wide. Later in the gradient, a more diffuse layer of water approximately 11Å wide will be formed\(^{317}\). Interactions between these layers and the polar analyte produces retention HILIC is therefore more similar to RPLC, as this is also a method related to liquid-liquid chromatography that uses polar mobile phases\(^{315}\). HILIC and RPLC differ in the polarity of the stationary phases that are used\(^{315}\). The more hydrophilic the analyte, the greater the interaction between the analyte and the immobilised layer of water and the greater the retention of the analyte. Greater retention is seen when the mobile phase is less polar, as a HILIC gradient method progresses, the proportion of the water content in the mobile phase increases causing the immobilised layer of water to grow; the growing immobilised layer reduces the
tightness to the stationary phase and the analyte is retained less \(^ {310}\). This expansion of the water layer and the effect on retention is shown in Figure 61.

![Figure 61: A representation of the effect of the percentage of water on the presence of the immobilised layer of water. The immobilised layer is represented by the blue area. At low aqueous percentage the immobilised layer is tight to the column stationary phase and analyte is retained. As the percentage of water increases the immobilised layer expands and the analyte interacts more with the components of the mobile phase flowing through the column.](image)

HILIC in carbohydrate analysis use aqueous mobile phases typically composed of aqueous solutions of ammonium formate, ammonium acetate or ammonium bicarbonate \(^ {318}\). These salts are used due to their high solubility in both organic and aqueous solvents. The second solvent composing the mobile phase is normally an aprotic organic solvent, typically MeCN \(^ {319}\). This solvent system means samples can be detected with FLD and are suitably volatile for detection in MS \(^ {319}\). The compatibility of HILIC with these common analytical techniques has meant HILIC has dominated the field of carbohydrate analysis. However, HILIC does suffer from limitations which are discussed in the oncoming sections \(^ {195,320}\).

4.1.1 Limitations of HILIC-HPLC chromatography
The limitations that HILIC suffer from are varied. Some HILIC methods suffer from poor peak shape which may cause difficulties in method development. HILIC methods generally suffer from long equilibration times (on some occasions these can range to 20 CV) and HILIC also commonly suffers from irreversible sorption. Rarer issues associated with HILIC are due to the reliance on one solvent in the mobile phase. In the late 2000s, the global shortage of MeCN meant the ability to perform HILIC was disrupted.

4.1.1.1 Effect of mobile phase pH and salt composition on HILIC-HPLC

Imperfect peak shapes are a known issue in HILIC, and it is common to see traces showing some degree of peak tailing or peak fronting. Peak fronting is a form of peak asymmetry, where peaks have longer front halves compared to back halves. Whereas a tailing peak is the opposite of this where the asymmetry seen in a longer back half of the peak compared to the front (Figure 62).

![Figure 62: An example of an ‘ideal’ peak (left), a fronting peak (middle) and a tailing peak (right).](image)

Peak shapes in HILIC are affected greatly by the ionic strength of buffers. Salts are added to the mobile phases in HPLC to control or promote electrostatic interactions between analytes and stationary phases. The changes in salts concentrations are known to affect elution in HILIC. Typical salt concentrations range from 5-100 mM and are added to give better reproducibility and peak shape. It is recommended to maintain a minimum buffer concentration of 5 mM in the final mobile phase at 95% MeCN.
Salts in mobile phases alter retention due to the effect the salts have on the immobilised layer of water with the effect on the immobilised layer determined by the type of salt used. Differences in the effect on the layer depend on whether they are kosmotropic or chaotropic. Kosmotropic salts are those that cause strict strongly held spheres of hydration and promote hydrogen bonding. Hydrogen bonding contributes to the overall mechanism of separation. Kosmotropes are high in the Hofmeister series and include citrates, tartrates, sulphates and phosphates. On the other side of the Hofmeister series are chaotropes, these are salts that antagonize hydrogen bonding, have thin weakly held spheres of hydration and include trifluoroacetates, perchlorates and iodides. At higher concentrations, chaotropes reduce retention of eluites (the eluted solute) while addition of kosmotropes increase retention.

Alpert et al (2018) investigated the effect of strong and weak kosmotropes and strong and weak chaotropes on retention in HILIC. If it were to follow the expected pattern, the highest retention should be seen with the strongest kosmotrope and this was the case. Changing the salt to the weak kosmotropic anion formate resulted in a large decrease in retention. A smaller decrease in retention was encountered in the mobile phases containing the weakly chaotropic anion bromide, while only a small difference between weak chaotropes bromide and strong chaotropes perchlorate was observed. Alpert et al (2018) also investigated the effect of concentrations on retention. Concentrations between 5 and 120 mM were used to investigate the effect of kosmotropic salts on retention. The concentration of strong kosmotropic anion sulphates had a large effect. As the concentration increased between 5 and 40 mM an increase in retention was seen but subsequent concentration increases were accompanied with a decrease in retention. This observation may be explained by salting out, where high concentrations of kosmotropic salt sequesters water, the water molecules involved in the solvation of salt causes the solutes to partition into the non-aqueous phase, decreasing the attraction to immobilized layer with the consequence of decreased retention. The effect of increasing the concentration of the chaotrope perchlorate (5 - 120 mM) on
the retention of analytes did not show as large an effect as kosmotropic salts and a more gradual effect for decreasing retention was observed. The concentration of perchlorate however had a larger effect on the retention of positively charged analytes. Above concentration of 80 mM, a noticeable increase in retention was observed indicating that this change was not as a result of hydrogen bonding but more likely due to electrostatic interactions.

The pH of the mobile phase also affects the peak shape and retention of analyte in HILIC. The majority of HILIC separations are performed with mobile phases within the range of pH 3 – 8 due to the instability of silica-based columns outside of these ranges. The primary effect of pH in a mobile phase is to produce a charge on the analyte. It is preferable to transform analytes into their charged form as they are usually more hydrophilic than their neutral forms, thereby increasing retention. Alpert and co-workers investigated the effect of pH on retention by analysing cytosine and cytidine at pH 3 and 6. At pH 3 these molecules are positively charged, whereas at pH 6 they are uncharged. The presence of the positive charge increased retention time by 15 minutes for cytosine and 12 minutes for cytidine.

4.1.1.2 The effect of mobile phase solvents in HILIC-HPLC

The choice of mobile phase solvents must be considered during method development. As retention of an analyte is partly due to the composition and thickness of the water layers, separation relies on water partitioning from the bulk eluent. Any organic solvents used in this analysis must be miscible with water. Aprotic solvents without hydrogen donor or acceptor functionalities are most commonly used, as protic solvents compete with water in solvating the stationary phase, but protic solvents such as alcohols may be used. Alcoholic solvents result in reduced retention and are preferred in cases where there will be strong interactions with the column. Reduced retentivity is due to hydrogen bonding between the alcohol and the immobilized water layer producing competition between the two in solvating the stationary phase. Amongst alcohols, retention increases as the carbon chain of the alcohol increases or as the acidity of the
alcohol decreases \(^{310}\). MeCN is the most common organic solvent in HILIC as it satisfies these considerations \(^{328}\). The eluotropic series (defined as the power of mobile phase to elute materials from a given stationary phase) of common organic HILIC solvents is shown in Figure 63.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td><img src="image" alt="Structure of Acetone" /></td>
</tr>
<tr>
<td>Isopropanol</td>
<td><img src="image" alt="Structure of Isopropanol" /></td>
</tr>
<tr>
<td>Acetonitrile</td>
<td><img src="image" alt="Structure of Acetonitrile" /></td>
</tr>
<tr>
<td>Ethanol</td>
<td><img src="image" alt="Structure of Ethanol" /></td>
</tr>
<tr>
<td>Dioxane</td>
<td><img src="image" alt="Structure of Dioxane" /></td>
</tr>
<tr>
<td>DMF</td>
<td><img src="image" alt="Structure of DMF" /></td>
</tr>
<tr>
<td>Methanol</td>
<td><img src="image" alt="Structure of Methanol" /></td>
</tr>
<tr>
<td>Water</td>
<td><img src="image" alt="Structure of Water" /></td>
</tr>
</tbody>
</table>

*Figure 63*: The eluotropic series for commonly used organic solvents in HILIC \(^{195}\).

The composition of injection solvent also affects the peak efficiency, although this is not exclusive to HILIC. Matching the injection solvent with the method gradient is suggested to overcome these problems by nulling or reducing the inject solvent mismatch \(^{329}\). This may cause problems depending on the solubility of compounds, since highly polar compounds may suffer from poor solubility in a solvent system with high percentages of MeCN \(^{329}\).

4.1.1.3 Other common challenges of HILIC-HPLC

Another disadvantage particularly common in HILIC is column bleed \(^{330}\). Column bleed is where bound stationary phase disassociates ('bleeds') during analysis.
Column bleed is a prominent source of background signal in LC-MS analyses and reduce analyte ionisation as the released bonded phase is not volatile and contaminates analysis at the point of solvent evaporation. Column bleed is not a phenomenon exclusive to HILIC and is experienced with all stationary phases, but the effect is considered to be worse in HILIC columns.

Irreversible sorption is another issue that effects the retention in HILIC, this is where molecules are absorbed onto the stationary phase in such a way that they cannot diffuse or desorb from the surface. The diverse chemical functionality of polar compounds leads to complex interactions between analyte and the immobilised layer. In some cases certain structural features such as charged groups lead to strong secondary interactions between the column and analytes become sorbed onto the stationary phases. Irreversible sorption causes differences in retention as it effectively reduces the length of the column, this is illustrated in Figure 64. Absorption occurs on the first point of contact between the analyte and the stationary phase and prevents this section of column from interacting with the analyte, preventing separation in this region. This reduces the effective area of separation, and therefore reduces retention and separation as normal interactions only resume after the analyte has entered the area of column without irreversible sorption. The occurrence of this phenomenon differs depending on the HILIC stationary phase; irreversible sorption is more prevalent in amine silica and bare silica stationary phases but is not often seen in amide silica phases which are common in glycan analysis.
Figure 64: Representation of the effect of irreversible sorption. The top column shows the normal separation of analyte between the stationary phases. The bottom column shows the section at the beginning of the column with analyte irreversibly sorbed onto the column which affects separation and retention.

The column temperature has a minor impact on retention but HILIC is more sensitive to temperature than RPLC. A 4 °C increase in column temperature was the equivalent to approximately a 1% increase in MeCN concentrations. The effect of temperature is primarily related to the enthalpy of the analyte transfer between mobile and stationary phases. Generally in HILIC, increasing the temperature decreases retention, this phenomenon is explained by the movement of analyte from the bulk mobile phase at high MeCN gradients being an exothermic process, favoured at lower temperatures.

4.1.2 RP-HPLC chromatography methods of glycan analysis.

The common issues encountered with HILIC highlighted in the previous sections has meant alternatives to HILIC have been explored. RP-HPLC is used extensively in the analysis of peptides and proteins but is not commonly used for the analysis of glycans. The is due to RP mode chromatography having limited capacity for the retention of very polar compounds. The lack of retention is caused by solvophillic factors, there are few interactions between RP stationary phases and any polar functional groups and analytes produce favourable dipolar bonds with the solvent and elute in the void volume. However, the use of RPLC in analysing derivatised glycans is an emerging field with some benefits compared to HILIC.
4.1.3 Advantages of RP-HPLC for the analysis of labelled glycans

RP-HPLC results in faster elution times for analyses; this was shown in research by Saba et al (2001) who analysed N-linked glycans from ovalbumin labelled with PMP 12 in both HILIC and RPLC. In HILIC mode this analysis took 35 minutes however in RP it only required 6 minutes 336. RP also has the potential to improve detection of low abundance peaks compared to other methods. Figure 65 displays research results by Periat et al (2016). This study investigated the effect of column temperature on the retention and detector response of intact trastuzumab. A large difference in detector response was seen between the two modes, the peak in RPLC had a detection intensity of approximately 500 emission units (EU), while the peak in HILIC has an EU of approximately 80 337.
Figure 65: Comparison of the effect of temperature on the HPLC traces of intact trastuzumab with RPLC and HIIC at three temperatures. Reprinted with permission from Journal of Chromatography A \textsuperscript{338}.

Analysis of glycans by RP chromatography also benefits from less interaction between unlabelled sugars and stationary phase as retention is as a result of the added label \textsuperscript{330}. Underivatized glycans either elute as part of the void volume, or show very little retention and will be eluted early in the method \textsuperscript{193}. This benefits detection by MS avoiding any potential ion suppression effects. Ion suppression is where ESI efficiency is reduced due to other eluites being present reducing evaporation of the droplet \textsuperscript{339}. The coelution of labelled and unlabelled species in HILIC means that ion suppression may occur.
RPLC analysis also produces traces with fewer non-ideal peaks. Non-ideal peaks include split peaks, broad peaks, double peaks and multiple peaks. The analysis of a metabolite library with both HILIC and RP saw 65% of the peaks as “good peaks” in RP compared to 29% in the HILIC method, although this may also be due to the HILIC method requiring further optimisation.

4.1.4 Analysis of labelled glycans by RP-HPLC

The use of RP methods in the analysis of glycans is an emerging area, RP-HPLC has been used to analyse glycans derivatised by a variety of derivatisation methods including permethylation, Michael addition with PMP and reductive amination labelling with 2-AB, 2-PA, AMAC and ANTS. As RP-HPLC methods separate analytes on their relative hydrophobicity, it could be expected that the least hydrophobic glycans will elute first and the most hydrophobic species elute last. This means the largest glycans would elute first as they would have the lowest relative hydrophobicity. However, this is not always the case, and for some samples the weakly non-polar C-H bonds of the carbohydrate contribute to the retention which results in a different elution order.

HILIC methods of glycan analysis have established methods, but as RP glycan analysis is still emerging no universal method has been developed. Methods for RP-HPLC of glycans in the literature have been performed with a variety of mobile phases. Amongst the most common are acidic or neutral mixtures of water with acidic or neutral solutions of MeCN or MeOH with very shallow gradients of organic solvents.

The main stationary phase of choice in this analysis is C$_{18}$, however polar embedded RP columns have also been used. The polar embedded stationary phase differs to normal C$_{18}$ functionalised silica by containing a polar group (such as a carbamate) in the carbon chain. The polarity of this group in the stationary phase benefits the
interactions between the stationary phase and the analyte producing greater retention.

4.2 Aims

The use of RP-HPLC is a promising area for glycan analysis and many examples of RP analysis for labelled glycans have recently appeared in the literature. However, very little of this analysis has been performed with ProA 21 labelled glycans. This is despite the fact that in HILIC analysis of glycans ProA 21 has been shown to have superior performance in FLD and ESI-MS detection. The work in this chapter details the steps towards developing a method for the separation and analysis of glycans derivatised with ProA 21 and its alkyne derivative 35 by RP-HPLC. The methods used and the labels employed in this section are shown in the flowchart in Figure 66.
Figure 66: A flowchart showing the prospective order of research in this Chapter highlighting the methods and labels employed.
4.3 Results and discussion

4.3.1 Establishing conditions for RP-HPLC-FLD analysis of labelled glycans

The wide variety of method parameters, stationary phases and solvents that can be used in HPLC makes the development of an optimal method a complex task. Method development follows a process constructed around the target end analysis. First, the initial systems, the method of detection and the mode of HPLC are selected. This may also include the method of detection chosen. In this case both FLD and MS were selected. FLD was selected as this would provide a method of comparing this analysis to the HILIC analysis in Chapter 3 and was available in house, with MS available through collaborators.

Next, the type of method was considered. In this case the method selected from the beginning was RPLC as it is the most commonly utilised mode of HPLC in analytical laboratories as it has inherent advantages compared to HILIC as mentioned in previous sections. Previous research in this area has been performed with gradient mobile phase methods. A gradient method was selected for this method as gradient methods are commonly selected when the pool samples contain a large number of analytes. Glycan samples often contain over 20 analytes making a gradient method the logical way to proceed. Once a gradient method was selected, the nature and composition of the mobile phases could be chosen. Previous literature in this area has demonstrated the reliance of a gradient composed of a low percentage of organic. The organic solvents that dominate this analysis are MeCN, MeOH and 1-butanol with low concentrations of volatile acids, such as FA, acetic acid, TFA or ammonium acetate and ammonium formate. For this analysis, mobile phases were composed of 0.1% FA (aq) as solvent A and 99.9% MeCN with 0.1% FA as solvent B. These solvents and additives were chosen as they are directly compatible with LC-MS analysis. Finally, the column was selected. This analysis required a column stable at high aqueous conditions. This influenced the choice of column that could be adopted. In the end, the Phenomenex aqua® C18
column was selected as this column was reported to be stable at 100% aqueous conditions.

4.3.2 Developing a RP-HPLC-FLD gradient capable of analysing ProA 21 and 2-AB 10 labelled glycans

Following the selection of starting parameters, suitable gradient conditions could be explored for method optimisation. The analysis of a labelled GHP in HILIC is common as retention standards. These standards are assigned GU values which are compared to native glycans \(^\text{341}\). Retention standards in RPLC is traditionally an arabinose ladder which is an \(\alpha\)-1,5-linked arabinose homopolymer. This ladder is used rather than a glucose ladder \(^\text{342}\). GHP ladder was used in this case as it provided the ability of direct comparison between RPLC and HILIC methods.

Previous work in this area had highlighted that these methods required very low percentages of organic solvents, therefore the first method trialled had an initial gradient of 1% solvent B increasing to 10% over 30 minutes. The injection of ProA 21 labelled GHP with this method is shown in Figure 67.

![Figure 67: RP-HPLC-FLD trace of ProA 21 labelled GHP produced by acid hydrolysis of dextran.](image)

From Figure 67 it was immediately apparent that the order of elution was the reverse to the order predicted with low GU carbohydrates eluting sooner than the
higher GU. This elution order had previously been observed when analysing 2-PA 14 labelled glycans by RPLC. The method resulted in extreme coelution in the higher GU species, but it was felt that this first injection showed potential and provided a good starting point for further development of the HPLC method.

In developing this first method, the aims were to reduce the coelution of unresolved peaks at approximately 30 minutes retention time. This was attempted by slowing the increase in gradient of solvent B. This gradient started with an increase of 1-3% of solvent B over 35 minutes before increasing to 4% solvent B at 80 minutes. Figure 68 (a) is the response produced with this method. Peaks corresponding to the low GU analytes before 40 minutes were well resolved and separated. This method reduced some of the coelution observed in the first method and a small increase in resolution was seen but the coeluting hump was still present at approximately 42 minutes. The next development of the method aimed at reducing this coelution whilst also reducing the time to elute the first peak.
The next stage in mobile phase method optimisation in Figure 68 (b) consisted of a gradient of 1-3.4% solvent B over 35 minutes, before increasing to 3.8% solvent B by 65 minutes then increasing to 4% B at 100 minutes. A higher injection of labelled GHP (5 µg) was used for this analysis rather than 1 µg that was used for Figure 68 (a) to allow better visualisation of separation for the low abundant peaks at higher GUs. This method gave more of the expected separation and no noticeable coelution for the high GU analytes was observed. The elution time for the first peak under these conditions was 6 minutes earlier with a retention time of 10 minutes.
which could be reduced further. A large signal was seen in the conditioning step, this may indicate analyte is eluting in this step. Other issues encountered were the large peak widths, with some widths of 3 minutes, although this may be attributable to the higher injection volume.

This next method in **Figure 68 (c)** had a gradient initially starting at 1% to 3.2% of solvent B at 42 minutes before increasing to 3.4% at 60 minutes then finally increasing to 3.6% solvent B at 90 minutes. This method produced a trace with a peak distribution more similar to the HILIC method and closer to the desired outcome.

Further attempts at method development aimed to decrease the time to elute the first peak. This development led to the final gradient method. This gradient started at 3% solvent B; starting at this higher gradient should provide the earlier elution desired. The gradient increased to 3.5% of solvent B over 35 minutes before further increasing to 3.8% at 80 minutes. **Figure 68 (d)** is a trace of an injection with this method. This mobile phase saw reduced resolution of the low GU species compared to **Figure 68 (c)** but did result in the lower peak widths and earlier elution of the first peak that was desired.

For the RP analysis of 2-AB 10 labelled GHP, it was predicted that the method developed for ProA 21 would not be directly applicable. As retention is dependent on the hydrophobicity of the label and as 2-AB 10 is a more hydrophilic label it was expected that this method would not produce the same results. The partition coefficient or logP of a molecule provides a measure of hydrophobicity. The higher the value the more hydrophobic the molecule; 2-AB 10 has a logP of 0.3 compared to 0.95 for ProA 21 which would result in a significant change in retention. As 2-AB 10 is a less hydrophobic molecule, a lower proportion of organic solvent may be required in the gradient to produce the same separation as there will be a weaker interaction between the label and the stationary phase. This was the rationale behind why 2-AB 10 was employed: to investigate the impact that the label had on retention.
Again, initially unsure of how low the gradient of solvent B would be necessary, the first method of analysis started at 1% solvent B before rising to 3.2% at 42 minutes then rising to 3.4% at 60 minutes and finally rising to 3.6% at 90 minutes. **Figure 69 (a)** shows the FLD trace of the first HPLC method for RP analysis of 2-AB 10 labelled GHP.
Figure 69: Method development of RP separation of GHP labelled with 2-AB 10.

This trace showed the same elution order as was seen with the ProA 21 labelled glycans (i.e. low GUs to high). This initial method suffered from late elution, only 8 peaks were eluted before the method reached the conditioning step at 90 minutes where a large increase in signal was associated with the rest of the pool eluting. The first peak did not elute until approximately 17 minutes where there was then a 20-minute gap between peaks at GU2 and GU3. This result suggests that 2-AB 10 labelled glycans are more strongly retained than ProA 21 labelled glycans. It appears that comparison of the logP values for the unconjugated labels 2-AB 10 and ProA 21 does not correctly reflect the comparative retention of glycans conjugated to these molecules. Furthermore, stronger retention of 2-AB 10 over ProA 21 labelled glycans has been described previously in the literature.

The development of this method therefore required a higher gradient of solvent B and the next method started at 1% solvent B before rising to 3.2% at 35 minutes, then increasing to 4.1% solvent B at 52 minutes before rising to 4.5% at 75 minutes. This method was used in the injection of Figure 69 (b). The result was the earlier elution of low GU peaks that was desired, but this method suffered from coelution of high GU peaks above 60 minutes. The next development aimed at reducing the coelution of the high GU peaks in the last trace. This method started at 1% solvent B before rising to 3.5% at 40 minutes, then gradually increasing to 4% at 70 minutes which was held until 90 minutes. This method was used in the injection in Figure 69 (c) and produced the well resolved peaks in the early stages of the method and...
better separation in the higher GU species. Improvement could still be made in subsequent methods by reducing the gaps between the peaks at 34 minutes and 52 minutes which unnecessarily lengthened the analysis. The final method used in Figure 69 (d) started at 2% solvent B which increased to 3.5% at 30 minutes, before rising to 4% at 80 minutes. This method reduced the large gaps between peaks GU6 and GU8 but did suffer from lower separation from the peaks corresponding to GU1 and GU2. This low resolution was not considered an issue though as low GU species are not likely to be the target of this analysis as smaller carbohydrates are normally analysed using isocratic elution conditions.

The development and optimisation of these methods on the labelled GHP samples in the previous section appeared to produce methods capable of separating analytes depending on GU. Analysis of the labelled carbohydrate could then be used to investigate the validity of these methods. Samples of labelled GHP were injected on the developed methods and results compared. Figure 70 (a) shows the RP-HPLC analysis of ProA 21 labelled GHP which acted as a control to other labels. This method was also applied to the analysis of ProA derivative 35 labelled glycans. Due to the nature of RP-HPLC being impacted largely by the properties of the label, the highly similar structures of the two labels were thought to result in similar retention and separation. The analysis of derivative 35 labelled GHP with this method can be seen in Figure 70 (b).
Figure 70: RP analysis of GHP labelled with a) ProA 21 and b) alkyne derivative 35.

Both of these traces showed a large degree of similarity to each other as expected due to the structural similarities of the two labels. Less expected was the similarities between these traces and the traces produced in the HILIC analysis of the same samples in Figure 44. In Figure 70 (a) it was possible to discern 32 GU whereas in Figure 70 (b) it was possible to discern 20 GU before poor selectivity prevented further characterization, a small increase compared to ProA 21. The elution order and pattern of the peaks closely resemble HILIC, however the peak sharpness and resolution at higher GU appeared greater in HILIC. In RP these high GU peaks appeared flatter and wider. The analysis of 2-AB 10 labelled GHP with both HILIC and RPLC is shown in Figure 71 (a) and (b).
Figure 71: HPLC analysis of 2-AB 10 labelled GHP performed by a) RPLC and b) HILIC.

The similarities of the elution order and the pattern of elution between the two modes can also be observed here. This analysis was also consistent with labels 21 and 35. A total of 29 GU were detected with HILIC compared to the 25 GU could be detected in RPLC. Once again, the wider and flatter peaks at higher GU prevented their detection.

Analysis of these samples with the methods developed in the previous section were successful in separating the simple carbohydrate polymers in RP but for a method to achieve widespread adoption it must be validated which evaluates and confirms whether the method is appropriate for its intended analysis. Criteria for method validation include precision, resolution and accuracy but many aspects of method
validation exist, depending on the purpose of the desired end analysis. The first of these aspects is precision, the precision of a method determines the degree of agreement amongst analysis. Precision is a component of three aspects: repeatability, intermediate precision and reproducibility.\textsuperscript{345} Repeatability is the variation experienced by a single analyst on a single instrument, this is commonly evaluated by performing replicates of the analysis. Intermediate precision is the variations encountered on different days, different machines and different analysts.\textsuperscript{345} This is similar to the robustness of a method, which is defined as the ability for an analytical method to be unaffected by variations or changes in samples or HPLC conditions and indicates the reliability in normal usage. Reproducibility is conformity of results between different laboratories evaluated in multi-laboratory studies. A precise method produces similar results across multiple analyses, analysts and systems. Precision can be tested by comparing multiple injections of the same sample and can be shown as relative standard deviation calculated by 
\[
\%RSD = \frac{\text{std dev}}{\text{mean}} \times 100\text{.}\textsuperscript{345}
\]

The LOD and limit of quantitation (LOQ) are also used to validate a method. As mentioned previously, the LOD is the lowest amount of analyte in a sample that can be detected (at a defined signal to noise ratio) so the presence of an analyte can be confirmed. The LOD is calculated as a ratio of signal to noise as $S/N$ determined by the equation $s = \frac{H}{h}$ where $H$ is the height of the peak and $h$ is the value of the noise fluctuation of the baseline in a chromatogram. The LOD of a method is usually defined at a ratio of 3:1. The LOQ is similar to LOD however the LOQ is the response where the peak can be quantitated to an exact value to an accuracy of \pm 20\% determined by a S/N ratio of 10:1.\textsuperscript{345}

Specificity and resolution are other important method validation parameters. The specificity of a method is the ability to unequivocally determine the presence of an analyte against other species that are expected to be present such as impurities, degradation products and pharmaceutical excipients.\textsuperscript{345} Resolution describes the separation power of the system relative to components of the mixture. High
resolution promotes specificity by giving the ability to distinguish peaks from each other. The resolution of two peaks is calculated by \( R_s = \frac{t_{R2} - t_{R1}}{\sqrt{w_1 + w_2}} \) where \( t_R \) are the retention times of the two peaks and \( w \), the peak widths. A resolution of more than 1.5 indicates a separation of two peaks. Finally, accuracy is the agreement of a result generated by a method to its true value. These analytical parameters are summarised in Table 7.

Table 7: Summary of analytical terms

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precision</td>
<td>The degree of agreement for each test result when applied to multiple analyses</td>
</tr>
<tr>
<td>Repeatability</td>
<td>The variation experienced during analysis performed by a single analyst on a single instrument</td>
</tr>
<tr>
<td>Intermediate precision</td>
<td>The variations in the analysis encountered from different times, analysts and instruments</td>
</tr>
<tr>
<td>Reproducibility</td>
<td>The variation in results between different laboratories</td>
</tr>
<tr>
<td>LLOD</td>
<td>The lowest response of analyte in a sample that can be detected so the presence of an analyte can be confirmed at a defined S/N ratio of 3:1</td>
</tr>
<tr>
<td>LOD</td>
<td>The lowest response of analyte that can be quantitated to an exact value to an accuracy of ±20% determined by a S/N ratio of 10:1</td>
</tr>
<tr>
<td>Specificity</td>
<td>The ability to accurately measure an analyte in the presence of interferences</td>
</tr>
<tr>
<td>Resolution</td>
<td>The measure of separation for two peaks at different retention times</td>
</tr>
<tr>
<td>Accuracy</td>
<td>The agreement of a result generated by a method to its true value</td>
</tr>
</tbody>
</table>

Samples were analysed with this developed method in triplicate and conformity of injections were compared. Table 8 details the retention time, peak area, peak height and peak width of the three injections for three peaks, one at a lower GU value, in this case GU5 was selected, one at a higher GU value, GU15, and one in-
between, in this case GU10. These peaks are annotated in Figures 70 and 71 (a). These data points were selected as they could be used to indicate the repeatability of the method. The RSD of these data points were calculated to see the degree of conformity between the injections. Peak width was determined to calculate resolution and the peak height was also determined to calculate signal to noise ratio.
Table 8: Evaluation of variation between injection for RP analysis of labelled GHP. Values are given to 1 decimal place (dp).

<table>
<thead>
<tr>
<th>Replicate</th>
<th>ProA 21 GU5</th>
<th>ProA 21 GU10</th>
<th>ProA 21 GU15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8.7</td>
<td>14.7</td>
<td>20.7</td>
</tr>
<tr>
<td>Retention</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>8.9</td>
<td>15.1</td>
<td>21.4</td>
</tr>
<tr>
<td>(RTNT)</td>
<td>8.7</td>
<td>14.9</td>
<td>21.2</td>
</tr>
<tr>
<td>Relative</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Standard</td>
<td>1.3</td>
<td>1.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Deviation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(RSD) of</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>retention</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>time</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak Area</td>
<td>11,613,209</td>
<td>9,101,201</td>
<td>4,495,768</td>
</tr>
<tr>
<td>RSD of</td>
<td>1.0</td>
<td>3.9</td>
<td>1.7</td>
</tr>
<tr>
<td>peak area</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak</td>
<td>1.0</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Width</td>
<td></td>
<td>1.3</td>
<td>1.3</td>
</tr>
<tr>
<td>Resolution</td>
<td>4.1</td>
<td>6.3</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>35 GU5</td>
<td>35 GU10</td>
<td>35 GU15</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>Peak Height</td>
<td>539,809</td>
<td>514,875</td>
<td>523,360</td>
</tr>
<tr>
<td>RSD of peak height</td>
<td>2.4</td>
<td>2.4</td>
<td>4.2</td>
</tr>
<tr>
<td>Signal to noise (S/N)</td>
<td>235.0</td>
<td>136.0</td>
<td>106.9</td>
</tr>
<tr>
<td>Replicate</td>
<td>1 2 3</td>
<td>1 2 3</td>
<td>1 2 3</td>
</tr>
<tr>
<td>RTNT</td>
<td>9.3 9.0 8.9</td>
<td>15.5 15.2 15.9</td>
<td>21.8 21.3 21.8</td>
</tr>
<tr>
<td>RSD of RTNT</td>
<td>2.0</td>
<td>2.4</td>
<td>1.4</td>
</tr>
<tr>
<td>RSD of peak area</td>
<td>0.7</td>
<td>9.9</td>
<td>4.7</td>
</tr>
<tr>
<td>Peak Width</td>
<td>1.1</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Resolution</td>
<td>5.0</td>
<td>4.8</td>
<td>4.4</td>
</tr>
<tr>
<td>Peak Height</td>
<td>2-AB 10 GU5</td>
<td>2-AB 10 GU10</td>
<td>2-AB 10 GU15</td>
</tr>
<tr>
<td>------------</td>
<td>-----------</td>
<td>-------------</td>
<td>-------------</td>
</tr>
<tr>
<td>4,174,501</td>
<td>4,214,382</td>
<td>4,286,402</td>
<td>653,282</td>
</tr>
<tr>
<td>RSD of peak height</td>
<td>1.3</td>
<td>3.9</td>
<td>5.1</td>
</tr>
<tr>
<td>S/N</td>
<td>2,632.1</td>
<td>4,844.1</td>
<td>4,865.4</td>
</tr>
<tr>
<td>RT NT</td>
<td>15.9</td>
<td>16.1</td>
<td>16.3</td>
</tr>
<tr>
<td>RSD of RTNT</td>
<td>1.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak Area</td>
<td>24,195,148</td>
<td>24,259,792</td>
<td>24,313,692</td>
</tr>
<tr>
<td>RSD of peak area</td>
<td>0.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak Width</td>
<td>1.9</td>
<td>2.1</td>
<td>2.2</td>
</tr>
<tr>
<td>Resolution</td>
<td>25.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak Height</td>
<td>745,349</td>
<td>729,319</td>
<td>726,029</td>
</tr>
<tr>
<td>-------------</td>
<td>---------</td>
<td>---------</td>
<td>---------</td>
</tr>
<tr>
<td>RSD of peak height</td>
<td>1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S/N</td>
<td>1,381.1</td>
<td>1,047.6</td>
<td>3,123.7</td>
</tr>
</tbody>
</table>
From Table 8, the repeatability of the retention times between injections for ProA 21 samples are similar between the injections. This is seen in the low RSD values of 1.3, 1.1 and 1.5 for the replicates of the three species, labelled GU5, GU10 and GU15. Although there is no rule of thumb for RSD per se where at “n” value the variation of the system is deemed satisfactory, lower values of RSD show less variance between the values. Label 35 also showed low RSD values at 2.0, 2.4 and 1.4 for the replicates of the three species, labelled GU5, GU10 and GU15. 2-AB 10 had the highest conformity seen by the lowest RSD at all three GU values at 1.1, 0.8 and 0.4 for the labelled GU5, GU10 and GU15 species.

The conformity in peak area for the three peaks were high for each of the three labels. The ProA 21 labelled species also had low RSD values at 1.0, 3.9 and 1.7. Label 35 also showed less similarity in peak area especially for the labelled GU10 with RSD values ranging from 0.7, 9.9, 4.7. The RSD for peak area for the 2-AB 10 labelled carbohydrates were the most similar of the three labels, particularly at the lower GU peaks. RSD values of peak area were 0.2 and 0.8 for the GU5 and GU10 species with a slight increase at 2.2 for the peak area of the GU15 species.

The resolution of the peaks for each label were calculated, all of which were satisfactory with values above 1.5. A trend was observed of resolution increasing as GU increased. The signal to noise was calculated, these values all showed these peaks were at a suitable intensity to allow quantitation. More variability was seen in these values due to fluctuation in noise.

4.3.3 RP-HPLC-FLD analysis of 21, 35 and 10 labelled RNase B glycans

Following the analysis of derivatised GHP, the next logical step was the RP analysis of glycans from natural sources. Glycans from RNase B were again chosen for the same reasons as outlined in Chapter 3. The analysis of RNase B glycans derivatised with ProA 21 and derivative 35 is shown in Figure 72.
Figure 72: RPLC analysis of RNase B glycans labelled with a) ProA 21 and b) alkyne 35 and c) the HILIC analysis of RNase B glycans labelled with 21.

Figure 72 (a) shows the trace produced by the ProA 21 labelled sample and the species labelled with 35 is shown in Figure 72 (b). Both of these traces show the presence of three peaks. The analysis of these samples by HILIC in Figure 46,
showed the detection of five peaks. This change in number of peaks is likely due to
the differences in separation mechanism. In RP, separation occurs due to size and
degree of bisection\(^{165}\). In the analysis of labelled RNase B glycans with RP-HPLC, the
three peaks can be seen due to coelution of glycans with similar sizes. Addition of
monosaccharide increased the mass but does not necessarily increase the size of
the glycan due to bisection such as the case of the two glycans in Figure 73 which
results in coelution. This is further illustrated by the collisional cross section (CCS) of
high mannose glycans investigated by Struve et al (2015). The CCS of a molecule is
the area around a particle which another particle must be in order for collision to
occur. The research by Struve et al (2015) looked at the CCS for adducts of high
mannose glycans in ion mobility-MS. They discovered that the CCS of the high
mannose N-glycans were very similar, especially larger glycans (such as Man8 and
Man9)\(^{346}\).

![Figure 73: Comparison and size and structures of Man5 and Man6 glycans. Although
Man6 contains an additional mannose both are similar “size”.](image)

The use of FLD in this analysis does not allow determination of structure and it is
therefore impossible to characterize these glycans and determine which are
coeluting without further analysis by MS or by comparison with a standard. The next analysis was performed on the 2-AB 10 labelled RNase B glycans samples and can be seen in Figure 74.

Figure 74: Analysis of 2-AB 10 labelled glycans analysed with a) RP and b) HILIC.

A similar elution pattern can be seen to the species labelled with ProA 21 by the elution of the three peaks with coelution. This trace provides the ability to see more evidence of the coelution as a shoulder can be seen at the peak at 15 minutes in Figure 74 (a), although once again the exact species responsible for the coelution cannot be deduced easily without standards. All RNase B glycan samples were analysed in triplicate to evaluate the repeatability of the injections. This was determined by comparing the values of the first two peaks annotated as peak 1 and peak 2 in Figures 72 and 74 (a) and are plotted in Table 9.
Table 9: Evaluation of variation between injection for RP analysis of labelled RNase B glycans. Values are given to 1 dp.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>ProA 21 Peak 1</th>
<th>ProA 21 Peak 2</th>
</tr>
</thead>
<tbody>
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<td></td>
<td>RT</td>
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<tr>
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<td>2.0</td>
</tr>
<tr>
<td></td>
<td>50.1</td>
<td>36.8</td>
</tr>
<tr>
<td></td>
<td>3.7</td>
<td>4.3</td>
</tr>
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<td></td>
<td>102.5</td>
<td>104.0</td>
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</table>

<table>
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<tr>
<th>Replicate</th>
<th>35 Peak 1</th>
<th>35 Peak 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT</td>
<td>RSD of RTNT</td>
</tr>
<tr>
<td></td>
<td>15.6</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td>4.2</td>
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<td>0.7</td>
</tr>
<tr>
<td></td>
<td>1.7</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>2-AB 10 Peak 1</td>
<td>2-AB 10 Peak 2</td>
</tr>
<tr>
<td>------------------</td>
<td>---------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Resolution</td>
<td>34.3</td>
<td>31.5</td>
</tr>
<tr>
<td></td>
<td>30.7</td>
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</tr>
<tr>
<td></td>
<td>32.6</td>
<td>34.4</td>
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<td>180.2</td>
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<td></td>
<td>209.4</td>
<td>197.7</td>
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</tr>
<tr>
<td></td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>RTNT</td>
<td>14.5</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td>13.5</td>
<td>23.7</td>
</tr>
<tr>
<td></td>
<td>23.0</td>
<td>22.4</td>
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<tr>
<td>RSD of RTNT</td>
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<td>2.6</td>
</tr>
<tr>
<td>Peak Area</td>
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<td>4,410,693</td>
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<tr>
<td></td>
<td>4,443,002</td>
<td>9,327,261</td>
</tr>
<tr>
<td></td>
<td>8,951,926</td>
<td>9,097,255</td>
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<tr>
<td>RSD of peak area</td>
<td>4.6</td>
<td>2.1</td>
</tr>
<tr>
<td>Peak Width</td>
<td>2.2</td>
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<tr>
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</tr>
<tr>
<td></td>
<td>2.4</td>
<td>2.8</td>
</tr>
<tr>
<td>Resolution</td>
<td>45.7</td>
<td>43.9</td>
</tr>
<tr>
<td></td>
<td>42.8</td>
<td>44.3</td>
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<tr>
<td></td>
<td>40.7</td>
<td>43.9</td>
</tr>
<tr>
<td>Peak Height</td>
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<td>84,863</td>
</tr>
<tr>
<td></td>
<td>86,560</td>
<td>157,162</td>
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<tr>
<td>RSD of peak height</td>
<td>2.9</td>
<td>1.1</td>
</tr>
<tr>
<td>S/N</td>
<td>27.8</td>
<td>14.5</td>
</tr>
<tr>
<td></td>
<td>20.5</td>
<td>53.6</td>
</tr>
<tr>
<td></td>
<td>27.1</td>
<td>38.0</td>
</tr>
</tbody>
</table>
The variation in retention time for all the labels were low, ProA 21 and 2-AB 10 had similar RSD with 1.3 and 2.8 for peaks 1 and 2 of the ProA 21 labelled species and an RSD of 2.6 and 2.6 for peaks 1 and 2 of 2-AB 10 labelled species. The largest change in retention between injections was encountered with the samples labelled with 35 with RSD values of 4.3 and 4.2 for peaks 1 and 2. The change in peak area was lower for 35 though, with RSD values of 4.4 and 2.4 for peaks 1 and 2. While the 2-AB 10 and ProA 21 showed similar conformity with 35 in this regard with RSD values of 4.4 and 2.4 peaks 1 and 2 of the ProA 21 labelled species and 4.5 and 2.1 for peaks 1 and 2 for species labelled with 2-AB 10. Variation was lower for peak 2 in all cases.

Only three peaks are present in the traces of all these samples, instead of five that would be expected for these samples and that are observed in HILIC analysis. Modifying the gradient may have reduced coelution. For example, elongating the early part of the gradient is a potential solution as all peaks in this analysis elute before 25 minutes. However, rather than optimising the gradient, an alternative to reduce coelution was attempted which is detailed later in this Chapter.

4.3.4 Development of a RP-HPLC-FLD gradient for the analysis of 35 and 10 labelled IgG glycans.

The next analysis was performed on IgG labelled glycans as was also performed previously in Chapter 3. The same method was used in this analysis but the wider variety of glycans in this sample, including fucosylated and sialylated glycans meant there was uncertainty whether the method developed previously would be applicable to this sample. The predicted lack of applicability for this method was seen in the analysis of IgG glycans labelled with 35. The trace of this injection in Figure 75 (a) shows elution of many of the 13 IgG glycans seen in the HILIC analysis in the conditioning step after 80 minutes. This meant that although the method was suitable for the analysis of simple glucose polymers, it was not satisfactory for the analysis of a greater variety of glycans such as the oligomannose glycans or complex
glycans. It therefore appeared necessary to change the gradient of solvent B for this analysis.
Figure 75: Method development of a RP-HPLC method for the analysis of IgG glycans labelled with ProA derivative 35. e) shows the HILIC analysis of ProA 21 labelled IgG glycans.

Figure 75 (b) shows an injection with the first development of this method which started at 3% solvent B before rising to 3.5% at 40 minutes then increasing to 5% by 80 minutes before the conditioning step. This method reduced the elution of species in the conditioning step, but analytes still eluted late in the method. Further increasing the gradient of solvent B would reduce the elution time of these peaks and the next method aimed at this by increasing the gradient of solvent B. The gradient in this method started at 3% to 3.5% over 20 minutes, before increasing to 6% at 80 minutes, before a conditioning step at 100% B. Figure 75 (c) is the result of an injection with this method. The increase to 6% of solvent B in the latter stages of the method had provided the reduction in the elution time desired and no peaks could be seen eluting in the conditioning step. However, this method still had the first peak eluting at nearly 34 minutes which could be reduced to shorten the method.

The next development of this method aimed at reducing the time required for the elution of the first peak, and started with 3.5% solvent B before increasing to 5% at 80 minutes. This increased the concentration of solvent B early in the method but
reduced the steepness of the gradient later in the method. Figure 75 (d) shows an injection with this method with earlier elution of the first peak that had been desired. Method development was also performed for the 2-AB 10 labelled samples, this development started with the gradient produced for the 21 and 35 labelled species. The FLD traces of gradient optimisation performed are shown in Figure 76.

![Figure 76](image)

**Figure 76:** Method development of a RP-HPLC method for the analysis of IgG glycans labelled with 2-AB 10.

**Figure 76 (a)** shows the injection for the first development of this method which started at 2% solvent B before rising to 3.5% at 40 minutes then increasing to 4% by 70 minutes with a further increase to 5% B at 100 minutes before the conditioning step. This method produced a trace of well resolved peaks, but some peaks were clearly in the conditioning step. This method was also long at over 100 hundred minutes. The next development of the gradient aimed at stopping the elution of species in the conditioning step and reducing the length of the gradient. **Figure 76 (b)** shows the injection of this optimised gradient. This gradient starts at 2% solvent
B before rising to 3.5% at 30 minutes, then increasing to 3.8% by 55 minutes with a further increase to 4.8% B at 70 minutes.

4.3.5 RP-HPLC-FLD analysis of 21, 35 and 10 labelled IgG glycans

**Figure 77** shows the RP analysis of IgG glycans with ProA 21 and derivative 35. These traces closely resemble each other showing high similarity. Again, a clear difference in the elution pattern was seen compared to the same analysis in HILIC in **Figure 77 (c)**. IgG samples typically contain 13 glycans in high abundance, however only eight peaks can be seen in abundance in this trace with some low abundance peaks eluting earlier.
Figure 77: RP analysis of IgG glycans labelled with a) ProA 21 and b) derivative 35 and c) annotated trace of labelled IgG glycans in HILIC.

Previous analysis in this area has shown that glycans labelled with 2-AB 10 in RP analysis eluted in a consistent order. Chen et al (2007) observed that acidic sialylated glycans elute first followed by high mannose glycans such as glycans found in RNase B. The next to elute are complex glycans, with single antennary glycans eluting before the tri- and tetra-antennary glycans, followed by biantennary glycans which elute last. However, it is unknown if this observation is the cause of the elution pattern seen in these traces, as structural confirmation was not possible.

Typically, the most abundant glycans in IgG are the G0F or G1F glycans which elute early amongst the pool of glycans in the analysis with HILIC mode. In this analysis however, the most abundant peak appears in the middle of the pool, if this analysis were to follow the observations from Chen et al (2007), these peaks should elute towards the end of all the pool of analytes. However, as the most abundant peaks are in the middle of the run this elution pattern does not appear to be the case in this example.
A sample of IgG glycans labelled with 2-AB 10 were also analysed by RP-HPLC and can be seen in Figure 78 as well as the same analysed by HILIC. As the order of elution is influenced by the label, as well as the glycans, the change is associated with a change in elution compared to the previous two traces.

Figure 78: Analysis of IgG glycans labelled with 2-AB 10 in a) RP and b) HILIC HPLC.

The conformity of the methods used for these analyses were also evaluated. These samples were each injected in triplicate and three annotated peaks from chromatograms in Figure 77 (a), (b) and Figure 78 (a) were selected to investigate precision, repeatability and S/N. This data is shown in Table 10.
Table 10: Evaluation of variation between injection for RP analysis labelled IgG glycans. All values are reported to 1 dp.

<table>
<thead>
<tr>
<th>Replicate</th>
<th>ProA 21 Peak 1</th>
<th>ProA 21 Peak 2</th>
<th>ProA 21 Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTNT</td>
<td>21.3</td>
<td>21.8</td>
<td>21.3</td>
</tr>
<tr>
<td>RSD of RTNT</td>
<td>1.2</td>
<td>1.4</td>
<td>1.8</td>
</tr>
<tr>
<td>Peak Area</td>
<td>5,491,734</td>
<td>5,518,721</td>
<td>5,464,632</td>
</tr>
<tr>
<td>RSD of peak area</td>
<td>0.5</td>
<td>3.2</td>
<td>5.5</td>
</tr>
<tr>
<td>Peak Width</td>
<td>1.7</td>
<td>1.9</td>
<td>1.8</td>
</tr>
<tr>
<td>Resolution</td>
<td>22.3</td>
<td>24.4</td>
<td>21.6</td>
</tr>
<tr>
<td>Peak Height</td>
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<td>116,822</td>
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<td>RSD of peak height</td>
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<td>3.1</td>
<td>6.0</td>
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<tr>
<td>S/N</td>
<td>40.7</td>
<td>42.3</td>
<td>64.6</td>
</tr>
</tbody>
</table>

<table>
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<th>3S Peak 1</th>
<th>3S Peak 2</th>
<th>3S Peak 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTNT</td>
<td>23.7</td>
<td>23.5</td>
<td>24.0</td>
</tr>
<tr>
<td>RSD of RTNT</td>
<td>23.7</td>
<td>28.9</td>
<td>28.7</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td><strong>RSD of RTNT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak Area</td>
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<td>8,633,973</td>
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<tr>
<td>RSD of peak area</td>
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<td>4.0</td>
</tr>
<tr>
<td>Peak Height</td>
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<td>122,967</td>
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<td>RSD of peak height</td>
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<td>2.1</td>
<td>2.5</td>
</tr>
<tr>
<td>Peak Width</td>
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<td>2.0</td>
<td>2.3</td>
</tr>
<tr>
<td>Resolution</td>
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<td>22.2</td>
</tr>
<tr>
<td>S/N</td>
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<td>39.1</td>
<td>39.7</td>
</tr>
<tr>
<td></td>
<td>2-AB 10 Peak 1</td>
<td>2-AB 10 Peak 2</td>
<td>2-AB 10 Peak 3</td>
</tr>
<tr>
<td>Replicate</td>
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<td>2</td>
<td>3</td>
</tr>
<tr>
<td>RTNT</td>
<td>20.9</td>
<td>21.2</td>
<td>21.4</td>
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<tr>
<td>RSD of RTNT</td>
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<td>1.3</td>
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<tr>
<td>------------------</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td><strong>Peak Width</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>Resolution</strong></td>
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<td>18.0</td>
<td>20.1</td>
</tr>
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<td>8,965</td>
<td>9,255</td>
</tr>
<tr>
<td><strong>RSD of peak height</strong></td>
<td>1.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>S/N</strong></td>
<td>33.2</td>
<td>62.7</td>
<td>24.4</td>
</tr>
</tbody>
</table>
The conformity of the retention time for this analysis was high, ProA 21 had RSD values of 1.2, 1.4, 1.7 for each of the three selected peaks. The samples labelled with 35 had slightly more consistent retention times with RSD values of 1.0, 0.5, 0.4 for each of the three selected peaks. The samples labelled with 2-AB 10 had RSD values of 1.1, 0.5, 0.3. The consistency of the peak areas shows more variability, the ProA 21 labelled samples had RSD values of 0.5, 3.2 and 5.5 for peaks 1, 2 and 3. The 35 labelled sample did not have as high RSD values as ProA 21 and saw variation in peak area with RSD values of 3.3, 0.9 and 4.0 for peaks 1, 2 and 3. The 2-AB 10 labelled samples also showed a similar trend with RSD values of 4.4, 2.0, 1.9. Chromatographic resolution was once again higher than 1.5, greater resolution was seen for peaks 2 and 3 than peak 1 for all labels, and the signal to noise of each peak with each label were also determined as above 10, allowing quantitation.

4.3.6 Further improvements for the RP-HPLC-FLD analysis of 21, 35 and 10 labelled glycans

Although the ability to separate and detect these glycans was achieved successfully for the labelled GHP samples, it was only partially successful for the labelled RNase B and IgG glycans as specificity was not always possible due to coelution. Some analysis also suffered from variability between injections. In order to reduce the observed coelution and variations between injections and improve the RP-HPLC analysis further, a few parameters of the method were changed or adjusted.

In the currently used method, solvent B was a mixture of 99.9% MeCN and 0.1 FA%. However, going forward solvent B was modified to a solution of 10% MeCN and 0.1% FA in water. This change should benefit repeatability of the method through using a mobile phase with 10% MeCN. During the injection, the mobile phase is a proportion of both mobile phases solvents. Changing phase B to a 10% MeCN solution while keeping mobile phase A as 99.9% H₂O and 0.1% FA will hopefully cause less errors from mobile phase proportioning. When mobile phase B is composed completely of MeCN, any error when proportioning the gradient will
have a large effect on the composition of mobile phase inside the column. As the method does not require a gradient of MeCN over 10% at any point, a change to a 10% MeCN (aq) solution is possible and may reduce the impact of the variations that inevitably occur in gradient proportioning. This change was made to hopefully make traces more reproducible in repetitions.

The analysis was also converted onto a microbore column with a 150 mm column length and a 2.0 mm internal diameter (ID) column rather than the analytical column previously employed with a 75 mm length column and a 4.6 mm ID. This was due to how the efficiency of a HPLC method can be improved by increasing column lengths and by decreasing column ID. The smaller ID of a column benefits analysis by increasing detector response, when ID is decreased the response of the same analyte was improved two-fold [348]. This is caused by reducing band spread which in turn increases analyte concentration, as illustrated in Figure 79. Band spreading is a process where the analyte band widens in the column due to dilution which causes detection of wider peak and loss of sensitivity and resolution. A smaller column ID reduces band spreading within the column and provides a higher response in the detector.

![Figure 79: A demonstration on the effect of the ID of a column and peak elution.](image)
This smaller column with a smaller ID also had smaller particles. Reduction in column particle size means that methods can be performed at lower flow rates. A lower flow rate is required as the reduced particle size increases the surface area of the chromatographic material in the column. This means that pressure inside the column increases and a lower flow rate is used to reduce the pressure of the system that would otherwise damage the system. This reduces the quantity of mobile phase required to produce the high pressure necessary for separation which saves mobile phase. The flow rates for a 4.6 mm ID column with 5 μm particle size in this case was 1 mLmin⁻¹, whereas a 4.6 mm ID column with 3 μm particle size in this case was 0.35 mLmin⁻¹, with both producing similar pressure of the system. Reducing flow rates is beneficial for ionization in LC-MS due to the effect that flow rates have on droplet size. Lower flow rates cause smaller droplets which require fewer droplet fission events and less solvent evaporation before the ion is released into the gaseous phase 349.

The use of a longer column also gives increased separation due to the longer bed length of stationary phase increasing the amount of theoretical plates. When changing a method between columns with different lengths the method may need adjustment. In this case, the use of the longer column did require modification of the LC method to accommodate the increased column length and reduced ID.

4.3.7 Analysis of 21, 35 and 10 labelled GHP using optimised RP-HPLC-FLD conditions

After altering the method to produce a similar elution pattern as the previous column, the first samples injected were the samples of labelled GHP. The same samples of 21 and 35 labelled GHP in Figures 70 and 71 were injected and are shown in Figure 80.
Figure 80: RPLC analysis with 150mm length, 2.0mm ID column of GHP labelled with a) ProA 21, b) derivative 35 and c) 2-AB 10. d) shows the HILIC analysis of GHP labelled with 2-AB 10.

These injections showed clear similarity to the earlier injections, both the elution order and peak pattern were consistent with the larger ID column and with what was seen previously in HILIC mode analysis. These samples were injected in triplicate to investigate the precision of this method. This data is shown in Table 11.
Table 11: Evaluation of variation between injections for RPLC analysis (150mm length, 2.0mm ID column) of labelled GHP. All values are reported to 1 dp.

<table>
<thead>
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<th>ProA 21 GU10</th>
<th>ProA 21 GU15</th>
</tr>
</thead>
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<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td><strong>RT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NT</td>
<td>13.9</td>
<td>14.6</td>
<td>14.7</td>
</tr>
<tr>
<td>RSD of RTNT</td>
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<td></td>
</tr>
<tr>
<td><strong>Peak Area</strong></td>
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<td>36,619,613</td>
<td>35,809,471</td>
</tr>
<tr>
<td>RSD of peak area</td>
<td>1.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Peak Width</strong></td>
<td>2.6</td>
<td>2.7</td>
<td>2.6</td>
</tr>
<tr>
<td><strong>Resolution</strong></td>
<td>32.3</td>
<td>35.4</td>
<td>35.5</td>
</tr>
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<td><strong>Peak Height</strong></td>
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<tr>
<td>RSD of peak height</td>
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<td></td>
</tr>
<tr>
<td><strong>S/N</strong></td>
<td>306.0</td>
<td>375.5</td>
<td>333.6</td>
</tr>
</tbody>
</table>

35 GU5 | 35 GU10 | 35 GU15
<table>
<thead>
<tr>
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<th>2</th>
<th>3</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<td>15.1</td>
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<th>2-AB 10 GU10</th>
<th>2-AB 10 GU15</th>
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<tr>
<td>Peak Width</td>
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<tr>
<td>Resolution</td>
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<td>29.2</td>
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<td>892,320</td>
<td>880,391</td>
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<tr>
<td>S/N</td>
<td>4,358.0</td>
<td>4,131.1</td>
<td>12,227.7</td>
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The elution times of the GU5 peak was approximately 6 minutes later for ProA 21, 6.5 minutes later for derivative 35 and 2 minutes later for 2-AB 10 compared to the shorter column. The change in the mobile phase to a 10% mixture was hoped to result in greater repeatability for this analysis. However, the RSD values of the retention times was higher for the ProA 21 labelled samples with this organic phase compared with the previous organic phase, with values of 2.9, 2.6 and 1.9 compared to 1.3, 1.1 and 1.6 for the labelled GU5, GU10 and GU15 species. This means the change of the organic phase to 10% MeCN and 0.1% FA (aq), which was aimed to produce a more repeatable gradient leading to less variability, did not produce the desired effect in this case. However, this may instead be due to the change in the column and so it is impossible to confirm whether the change of the mobile phase had an effect or not.

The retention times for the 35 labelled samples had RSD values of 1.3, 1.6 and 1.7 with the new organic mobile phase column compared to 1.2, 1.6 and 1.7 for the labelled GU5, GU10 and GU15 species. These injections showed no noticeable improvement in variability. The analysis of the 2-AB 10 labelled samples showed a much-reduced RSD with values of 0.3, 0.2 and 0.1 for GU5, GU10 and GU15 compared to 1.1, 0.9 and 0.4 for the 2-AB 10 labelled GU5, GU10 and GU15 species with the column with a lower ID longer column and new organic phase. These low values indicate the repeatability of this analysis.

The variation in peak area did also not change for the ProA 21 labelled samples with RSD values of 1.2, 0.8 and 3.5 for the GU5, GU10 and GU15 species compared to 1.0, 3.9 and 1.7 that was seen previously. The expectation that reducing the ID would result in an increase in peak area was observed with the peak area for the ProA 21 labelled in this case as samples showed an approximate three-fold increase in peak area for all of the species. Peak area for the samples labelled with 35 also resulted in a two-fold increase in peak area, however this was only observed for the high GU analyte.
The RSD values of peak area for the 2-AB 10 labelled samples again did not show any noticeable improvement, with values 0.7, 0.7 and 1.1 compared to 0.3, 0.8 and 2.2 for the GU5, GU10 and GU15 species. A four-fold increase in peak area was seen between the analyses performed on the analytical (4.6 mm ID) and microbore (2.0 mm ID) chromatography columns, respectively.

The reduction of the column ID leading to sharper peaks should also improve the resolution of the peaks. For ProA 21, a large increase in resolution was observed, although a portion of this may be attributable to the new method rather than the impact of the new column alone. The 35 labelled samples also resulted in an increase in resolution for the low GU species. However, the peaks resulting for the GU15 species showed no change in resolution. The resolution of the 2-AB 10 samples were similar between the two analyses but resolution was more consistent. As resolution is expected to increase when changing to a column with lower ID the absence of change is likely attributable to the new gradient which could be improved with further development. Signal to noise ratios also were again sufficient to allow quantitation. However, no significant increase was seen with this method compared to the analysis with the column with an ID of 4.6 mm.

4.3.8 Analysis of 21, 35 and 10 labelled RNase B glycans using optimised RP-HPLC-FLD conditions

This same method was then used in the analysis of the same RNase B samples and these analyses are displayed in Figure 81. These traces also showed three peaks. However, the traces for ProA 21 and its derivative 35 showed more similarities to the trace produced previously with the 2-AB 10 labelled samples with the larger analytical ID column (4.6 mm) where the first peak has a clear visible shoulder, indicating improved chromatographic resolution.
Figure 81: RPLC analysis with 150mm length, 2.0mm ID column of RNase B glycans labelled with a) ProA 21, b) derivative 35 and c) 2-AB 10.

These samples were injected in triplicate to evaluate repeatability and to compare the two column IDs. This data is shown in Table 12.
Table 12: Evaluation of variation between injections for RPLC analysis (150mm length, 2.0mm ID column) of labelled RNase B glycans. All values are reported to 1 dp.

<table>
<thead>
<tr>
<th></th>
<th>ProA 21 Peak 1</th>
<th></th>
<th>ProA 21 Peak 2</th>
<th></th>
<th>ProA 21 Peak 2</th>
<th></th>
<th>ProA 21 Peak 2</th>
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<td>2</td>
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<td>3</td>
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<td>23.8</td>
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<td>30.1</td>
<td>30.3</td>
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<td>121,848,952</td>
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<td>3.2</td>
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<td></td>
<td></td>
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<tr>
<td>Peak Width</td>
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<td>3.5</td>
<td>4.2</td>
<td>3.8</td>
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<p>|               | 35 Peak 1 |               | 35 Peak 2 |               |               |               | 3             |
| Replicate     | 1         | 2             | 3         | 1              | 2             | 3              | 3             |</p>
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<th>Replicate 2</th>
<th>Replicate 3</th>
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<td>2.1</td>
<td>2.1</td>
<td>2.1</td>
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<td>2,515.8</td>
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<td>7,735.4</td>
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This method saw improved RSD for the retention times of the ProA 21 labelled sample. The two peaks had values of 0.4 and 0.4 for peak 1 and 2 compared to 1.3 and 2.8. This marked decrease in RSD for retention time was also seen for samples labelled with 35 at 0.6 and 0.6 compared to 4.3 and 4.2 for peaks 1 and 2. For 2-AB 10 labelled samples a large decrease in RSD was also seen at values of 0.4 and 0.4 compared to 2.6 and 2.6 for peaks 1 and 2.

An approximate three-fold increase in peak area was seen for all of the samples, a change in the RSD values were seen in the 2-AB 10 labelled samples 0.4 and 0.4 in this analysis compared to 4.6 and 2.1 seen previously for peaks 1 and 2. The resolution also increased for all samples, although the increase for the 2-AB 10 labelled samples was not as high as the other two labels. The S/N ratio of the peaks were all satisfactory: a large difference was seen in the S/N of the first injection compared to later injections as this trace had lower variation in the baseline.

4.3.9 Analysis of 21, 35 and 10 labelled IgG glycans using optimised RP-HPLC-FLD conditions

Following the separation of RNase B glycans, IgG glycans were again analysed with the new microbore (2.0 mm) chromatography column. This method also had to be modified to be compatible with the new column. The same samples of labelled IgG were injected, and the traces of these injections are shown in Figure 82.
Figure 82: RPLC analysis with 150mm length, 2.0mm ID column of IgG glycans with a) ProA 21, b) 35, and c) 2-AB 10.

The samples were injected in triplicate to compare the repeatability of this method (Table 13). The elution of the peaks in this method were inconsistent with the previous traces which meant it was impossible to directly compare the two analyses in the way that had been performed for the RNase B glycan samples.
Table 13: Evaluation of variation between injections for RPLC analysis (150mm length, 2.0mm ID column) of labelled IgG glycans. All values are reported to 1 dp.

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<tr>
<th>Replicate</th>
<th>ProA 21 Peak 1</th>
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<th>2-AB 10 Peak 3</th>
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</tr>
<tr>
<td>Peak Area</td>
<td>144,476,948</td>
<td>148,533,563</td>
<td>147,162,073</td>
</tr>
<tr>
<td>RSD of peak area</td>
<td>1.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak Width</td>
<td>1.3</td>
<td>1.5</td>
<td>1.4</td>
</tr>
<tr>
<td>Resolution</td>
<td>8.7</td>
<td>9.4</td>
<td>8.8</td>
</tr>
<tr>
<td>Peak Height</td>
<td>77,139</td>
<td>65,666</td>
<td>62,288</td>
</tr>
<tr>
<td>-------------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>RSD of peak height</td>
<td>11.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S/N</td>
<td>403.9</td>
<td>252.6</td>
<td>259.5</td>
</tr>
</tbody>
</table>
The IgG glycan samples labelled with ProA labels 21 and 35 all showed very similar retention times in the repeated injections and all had very low RSD demonstrating the very high degree of conformity with each injection. The ProA 21 labelled samples has RSD values of 0.0, 0.0 and 0.0 for GU5, GU10 and GU15. These low RSD illustrates the very close retention times between the three injections. For the 35 labelled samples RSD values were 0.0, 0.1 and 0.0 for peaks GU5, GU10 and GU15. The 2-AB 10 labelled IgG glycans also showed high conformity between injections with RSD values of 3.7, 2.9, 1.2 however these values indicate greater variation than seen with ProA 21 and derivative 35.

The peak areas also showed high conformity between each injection, the 21 labelled sample had RSD values of 1.1, 0.8 and 1.9 for GU5, GU10 and GU15. The 35 labelled samples had RSD values of 0.3, 1.6 and 3.8 for the GU5, GU10 and GU15 species. The 2-AB 10 labelled species saw similar RSD values for both peaks 1 and 2 at 1.4, 2.5 however the final peak resulted in a large change in RSD with a value of 16.3 which is seen on the third injection which may be caused by the presence of a contaminant in this injection. Chromatographic resolution was once again satisfactory for all labels with resolutions in every case higher than 1.5 and the S/N ratios for all injection with all labels were suitably high to allow quantitation.

4.3.10 RP-nano-LC-MS analysis of 21, 35 and 10 labelled GHP

The RP-HPLC with FLD detection presented in the previous sections of this chapter demonstrates that it is possible to make putative assignments of peaks observed in samples that were analysed with FLD. These assignments are primarily based on GU value in reference to the GHP standard. However, as mentioned previously, due to the nature of FLD detection these assignments are not irrefutable. To confirm the assignments of these eluets, the GHP samples were also analysed with MS detection. Furthermore, this allowed the observation of the behaviour of labelled glycans in MS.
In this analysis the samples were analysed by Nano-LC-MS. Nano-LC is a miniaturized LC method and functions in a similar way to standard HPLC. Differences between the two systems are encountered in the chromatographic material. Nano-LC uses an LC column of fused silica capillaries, these columns have ID of typically 75 μM, with particles in similar size range to HPLC (2-5 μm). Due to this smaller ID nano-LC systems are typically operated at flow rates of below 500 nLmin⁻¹. As mentioned previously, reducing the column ID results in less chromatographic dilution resulting in increased concentration of the sample increasing detection sensitivity. Downscaling a column from a 4.6 mm ID to 75 μm ID would be expected to result in a 4000-fold increase in sensitivity. Nano LC columns are available in lengths up to 75 cm and above which provides good separation of complex samples.

Differences between Nano-LC and HPLC are also encountered in the machinery. Nano LC is often performed with a column switching system with a trapping column and an analytical column which generally requires two pumps, the loading pump and the nano pump. The sample is injected and concentrated on the trapping column at a relatively high flow rate (typically 5-20 μLmin⁻¹). Any species in the sample that are not trapped are directed to waste, which means while concentrating the sample, this also removes salts which may produce negative effects in ESI such as ionisation quenching. While the trapping occurs, flow from the nano pump is also inline with the analytical nano column. The valve then switches and places the trapping column in line with the nano pump flow, eluting analytes from the trapping column and onto the nano-LC analytical column where they are separated and then go on to be detected by ESI-MS. This nano-LC set up is shown schematically in Figure 83.
GHP samples labelled with ProA 21 or alkyne derivative 35 were injected onto a nano LC system with a mobile phase composed of 99.9 % water, 0.1 % FA as solvent A and 99.9 % MeCN, 0.1 % FA as solvent B. This analysis was performed with a flow rate of 250 nLmin\(^{-1}\) (analytical column) and the following gradient conditions (Table 14).

**Table 14: Nano-LC method gradient used to analyse labelled GHP.**

<table>
<thead>
<tr>
<th>Time</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>0.2</td>
</tr>
<tr>
<td>2.0</td>
<td>0.2</td>
</tr>
<tr>
<td>36.0</td>
<td>5.0</td>
</tr>
<tr>
<td>50.0</td>
<td>35.0</td>
</tr>
<tr>
<td>50.1</td>
<td>90.0</td>
</tr>
<tr>
<td>55.0</td>
<td>90.0</td>
</tr>
<tr>
<td>55.1</td>
<td>0.2</td>
</tr>
<tr>
<td>70.0</td>
<td>0.2</td>
</tr>
</tbody>
</table>

MS detection was performed on a Thermo Scientific Linear Trap Quadrupole (LTQ) Orbitrap Velos mass spectrometer in positive ionisation mode with a m/z range of 350-2000. A panel of extracted ion chromatograms (EIC) of the ProA 21 labelled GHP is shown in **Figure 84.**
Figure 84: The EIC MS trace at each GU value of ProA 21 labelled GHP analysed with nano-LC-MS. The annotations on each trace indicate time.
Each trace show an EIC at each GU and demonstrate facile detection of the analytes in the GHP samples, as was encountered with RP-HPLC with FLD. Displaying the data as EICs has the advantage of utilising the specificity of the MS detector of the Orbitrap-based high mass resolution mass spectrometer. This trace appears to follow the data of the labelled GHP analysed with FLD. However, this trace cannot be compared directly to the RP-HPLC analyses described earlier using FLD due to the numerous changes between the RP-HPLC and nano-LC set ups and the different type of data analysis (FLD traces versus EICs).

This type of analysis provided some valuable information. It shows consistency between the elution of labelled carbohydrates with both RP-HPLC and RP-nano-LC observing the lower GU species eluting before the high GU species. The effective ionisation of labelled glycans by ESI was also demonstrated in sufficient efficiency to allow the molecular characterisation of the sample. As glycans are highly polar molecules, ionisation of the samples may be hindered due to the hydrophobic bias seen in MS. Ionisation is improved in nano-LC-MS due to the lower flow rates. Figure 85 shows summed MS spectra of ProA 21 labelled glycans with GU values representative of naturally occurring N-linked glycans in the m/z range between 750 – 1800 m/z and over 27 to 34 minutes retention time.
Figure 85: Nano-LC-MS spectrum of ProA 21 labelled GHP between m/z 750 – 1800.
The peaks corresponding to labelled GHP species have been highlighted. This shows the peaks resulting from the various analytes between m/z 750 – 1800 and displays a series of ions corresponding to glycans with increasing GU value. It also shows the propensity for large analytes to form doubly charged species. Doubly charged species start appearing at GU6 while no ions corresponding to the singly charged species are observed for GU values higher than GU7. These two figures have been compiled into Table 15 which shows the RTNT of each peak as well as the charge value z, theoretical m/z and observed m/z and ppm mass error between theoretical m/z and observed m/z.

**Table 15: Table of retention, theoretical mass, observed mass, ppm error and charge of ProA 21 labelled GHP.**

<table>
<thead>
<tr>
<th>Species</th>
<th>RTNT</th>
<th>Theoretical m/z</th>
<th>Observed m/z</th>
<th>ppm mass error</th>
<th>charge (z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GU 1</td>
<td>27.2</td>
<td>400.2442</td>
<td>400.2443</td>
<td>0.25</td>
<td>1</td>
</tr>
<tr>
<td>GU 2</td>
<td>27.5</td>
<td>562.2971</td>
<td>562.2971</td>
<td>0.00</td>
<td>1</td>
</tr>
<tr>
<td>GU 3</td>
<td>28.5</td>
<td>724.3500</td>
<td>724.3501</td>
<td>0.14</td>
<td>1</td>
</tr>
<tr>
<td>GU 4</td>
<td>29.0</td>
<td>886.4028</td>
<td>886.4029</td>
<td>0.11</td>
<td>1</td>
</tr>
<tr>
<td>GU 5</td>
<td>29.6</td>
<td>1,048.4560</td>
<td>1,048.4562</td>
<td>0.19</td>
<td>1</td>
</tr>
<tr>
<td>GU 6</td>
<td>30.2</td>
<td>1,210.5080</td>
<td>1,210.5093</td>
<td>1.07</td>
<td>1</td>
</tr>
<tr>
<td>GU 7</td>
<td>30.6</td>
<td>686.7842</td>
<td>686.7842</td>
<td>0.00</td>
<td>2</td>
</tr>
<tr>
<td>GU 8</td>
<td>31.1</td>
<td>767.8106</td>
<td>767.8108</td>
<td>0.26</td>
<td>2</td>
</tr>
<tr>
<td>GU 9</td>
<td>31.4</td>
<td>848.8370</td>
<td>848.8375</td>
<td>0.59</td>
<td>2</td>
</tr>
<tr>
<td>GU 10</td>
<td>31.6</td>
<td>929.8635</td>
<td>929.8641</td>
<td>0.65</td>
<td>2</td>
</tr>
<tr>
<td>GU 11</td>
<td>31.8</td>
<td>1,010.8900</td>
<td>1,010.8903</td>
<td>0.30</td>
<td>2</td>
</tr>
<tr>
<td>GU 12</td>
<td>32.0</td>
<td>1,091.9160</td>
<td>1,091.9171</td>
<td>1.01</td>
<td>2</td>
</tr>
<tr>
<td>GU 13</td>
<td>32.2</td>
<td>1,172.9430</td>
<td>1,172.9435</td>
<td>0.43</td>
<td>2</td>
</tr>
<tr>
<td>GU 14</td>
<td>32.4</td>
<td>1,253.9690</td>
<td>1,253.9698</td>
<td>0.64</td>
<td>2</td>
</tr>
<tr>
<td>GU 15</td>
<td>32.6</td>
<td>1,334.9960</td>
<td>1,334.9961</td>
<td>0.08</td>
<td>2</td>
</tr>
<tr>
<td>GU 16</td>
<td>33.0</td>
<td>1,416.0220</td>
<td>1,416.0234</td>
<td>0.99</td>
<td>2</td>
</tr>
<tr>
<td>GU 17</td>
<td>33.1</td>
<td>1,497.0480</td>
<td>1,497.0506</td>
<td>1.74</td>
<td>2</td>
</tr>
</tbody>
</table>
This data shows the small difference between the theoretical m/z of the species to the observed m/z expressed as ppm mass error (where 1ppm mass error corresponds to a mass deviation of 0.001%). A low ppm error demonstrates the mass accuracy of the system which then gives confidence in the correct assignment of peaks and thereby characterization of the sample. In this case, the peaks were all within a 2-ppm mass error at 4 decimal places indicating a good level of mass accuracy. For a well calibrated Orbitrap mass spectrometer a mass error <5 ppm would be expected. The analysis was also performed for the alkyne derivative labelled GHP, EICs of the first 20 GU can be seen in Figure 86.
Figure 86: The EIC MS trace at each GU value of alkyne 35 labelled GHP analysed with nano-LC-MS. The annotations on each trace indicate time.
Consistency in the elution of 35 labelled carbohydrates with both RP-HPLC and RP-nano-LC and between the two labels was again observed. The ionisation of the sample was again observed and enabled unambiguous assignment of individual ion m/z and GU values. Figure 87 shows summed MS spectra of 35 labelled glycans with GU values representative of naturally occurring N-linked glycans in the m/z range between 750 – 1800 m/z and over 27 to 34 minutes retention time.
Figure 87: Nano-LC-MS spectrum of alkyne derivative 35 labelled GHP between m/z 750 – 1800.
The peaks that correspond to the mass of labelled GHP species have been highlighted in the figure. Again, the propensity for larger analytes to form doubly charged species is observed. The data from these two figures have been compiled into Table 16.

### Table 16: Table of retention, theoretical mass, observed mass, ppm error and charge of derivative 35 labelled GHP.

<table>
<thead>
<tr>
<th>Species</th>
<th>RTNT</th>
<th>Theoretical m/z</th>
<th>Observed m/z</th>
<th>ppm mass error</th>
<th>charge z</th>
</tr>
</thead>
<tbody>
<tr>
<td>GU 1</td>
<td>29.1</td>
<td>410.2286</td>
<td>410.2286</td>
<td>0.00</td>
<td>1</td>
</tr>
<tr>
<td>GU 2</td>
<td>29.3</td>
<td>572.2815</td>
<td>572.2815</td>
<td>0.00</td>
<td>1</td>
</tr>
<tr>
<td>GU 3</td>
<td>30.5</td>
<td>734.3343</td>
<td>734.3344</td>
<td>0.14</td>
<td>1</td>
</tr>
<tr>
<td>GU 4</td>
<td>31.0</td>
<td>896.3871</td>
<td>896.3873</td>
<td>0.22</td>
<td>1</td>
</tr>
<tr>
<td>GU 5</td>
<td>31.7</td>
<td>1,058.4400</td>
<td>1,058.4400</td>
<td>0.38</td>
<td>1</td>
</tr>
<tr>
<td>GU 6</td>
<td>32.7</td>
<td>1,220.4930</td>
<td>1,220.4929</td>
<td>-0.08</td>
<td>1</td>
</tr>
<tr>
<td>GU 7</td>
<td>33.2</td>
<td>691.7764</td>
<td>691.7763</td>
<td>-0.14</td>
<td>2</td>
</tr>
<tr>
<td>GU 8</td>
<td>33.7</td>
<td>772.8028</td>
<td>772.8030</td>
<td>0.26</td>
<td>2</td>
</tr>
<tr>
<td>GU 9</td>
<td>34.1</td>
<td>853.8292</td>
<td>953.8294</td>
<td>0.23</td>
<td>2</td>
</tr>
<tr>
<td>GU 10</td>
<td>34.4</td>
<td>934.8556</td>
<td>934.8562</td>
<td>0.64</td>
<td>2</td>
</tr>
<tr>
<td>GU 11</td>
<td>34.7</td>
<td>1,015.8820</td>
<td>1,015.8826</td>
<td>0.59</td>
<td>2</td>
</tr>
<tr>
<td>GU 12</td>
<td>35.0</td>
<td>1,096.9080</td>
<td>1,096.9091</td>
<td>1.00</td>
<td>2</td>
</tr>
<tr>
<td>GU 13</td>
<td>35.2</td>
<td>1,177.9350</td>
<td>1,177.9358</td>
<td>0.68</td>
<td>2</td>
</tr>
<tr>
<td>GU 14</td>
<td>35.3</td>
<td>1,258.9610</td>
<td>1,258.9617</td>
<td>0.56</td>
<td>2</td>
</tr>
<tr>
<td>GU 15</td>
<td>35.5</td>
<td>1,339.9880</td>
<td>1,339.9881</td>
<td>0.01</td>
<td>2</td>
</tr>
<tr>
<td>GU 16</td>
<td>35.6</td>
<td>1,421.0140</td>
<td>1,421.0153</td>
<td>0.92</td>
<td>2</td>
</tr>
<tr>
<td>GU 17</td>
<td>35.7</td>
<td>1,502.0410</td>
<td>1,502.0421</td>
<td>0.73</td>
<td>2</td>
</tr>
<tr>
<td>GU 18</td>
<td>35.8</td>
<td>1,583.0670</td>
<td>1,583.0686</td>
<td>1.01</td>
<td>2</td>
</tr>
<tr>
<td>GU 19</td>
<td>35.9</td>
<td>1,664.0930</td>
<td>1,664.0960</td>
<td>1.80</td>
<td>2</td>
</tr>
<tr>
<td>GU 20</td>
<td>36.1</td>
<td>1,745.1200</td>
<td>1,745.1234</td>
<td>1.95</td>
<td>2</td>
</tr>
</tbody>
</table>
The elution of the peaks with this glycan ladder was approximately 2 minutes later than the corresponding ProA 21 labelled species. This data also shows high mass accuracy between the theoretical m/z of the species and the observed m/z, as was seen with the ProA 21 labelled species. In this case, the peaks were all within a 2 ppm mass error to 4 decimal places. As was also seen with the ProA 21 labelled species, relatively higher ppm error values were encountered for the higher m/z species. This observation may be explained by the fact that the mass resolution of the orbitrap mass analyser is dependent on the m/z value and decreases with increasing m/z. 355. Table 15 and 16 indicate that RP-nano-LC-MS analysis of labelled carbohydrates can be performed effectively and with high mass accuracy. It should be mentioned that triply charged ions for higher GU species were also observed in the nanoLC-MS analysis (data not presented) but these were of lower signal intensities.
4.4 Conclusions and future work

The work in this chapter has focused on developing methods for RP-HPLC analysis of labelled glycans. The method optimization in this chapter has led to the development of four methods that can be used in the analysis of a variety of glycan samples with a range of glycan labels. Method optimization was performed through variation of several HPLC conditions. Mobile phase gradient and column parameters such as column length, ID and particle size of RP silica packing material were evaluated and changed in the development. Buffer composition was also a target of optimisation with a change in organic buffer composition aimed at producing greater reliability from the method.

The most extensive route of optimisation pursued in this chapter was performed in the development of the gradient conditions which was the focus of much of the method development. Further development could have looked at examining the effect of column temperature on retention and the effect of acid present in the mobile phases, which could be investigated in future. A summary of the optimized methods is shown in Table 17 which shows the development of the method for analysis of labelled GHP samples and the positive or negative effects these changes had on the analysis.

Table 17: Comparison of the development of RP-HPLC methods used in the analysis of ProA 21 labelled GHP.

<table>
<thead>
<tr>
<th>Method name</th>
<th>HPLC Column</th>
<th>RTNT of GU 1</th>
<th>GU values assigned</th>
<th>Resolution of GU 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Phenomenex Aqua (75 mm × 4.6 mm, 5 μm particle and 125 Å pore size)</td>
<td>17.8</td>
<td>9</td>
<td>N/A</td>
</tr>
<tr>
<td>B</td>
<td>Phenomenex Aqua (75 mm × 4.6 mm, 5 μm particle and 125 Å pore size)</td>
<td>17.3</td>
<td>19</td>
<td>4.3</td>
</tr>
<tr>
<td>C</td>
<td>Phenomenex Aqua (75 mm × 4.6 mm, 5 μm particle and 125 Å pore size)</td>
<td>10.6</td>
<td>29</td>
<td>13.1</td>
</tr>
<tr>
<td></td>
<td>Phenomenex Aqua (75 mm × 4.6 mm, 5 μm particle and 125 Å pore size)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>5.5</td>
<td>33</td>
<td>23.5</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Phenomenex Aqua (75 mm × 4.6 mm, 5 μm particle and 125 Å pore size)</td>
<td>4.6</td>
<td>32</td>
<td>7.6</td>
</tr>
<tr>
<td>F</td>
<td>Phenomenex Aqua (150 mm × 2 mm, 3 μm particle and 125 Å pore size)</td>
<td>4.3</td>
<td>27</td>
<td>23.6</td>
</tr>
<tr>
<td>G</td>
<td>Phenomenex Aqua (150 mm × 2 mm, 3 μm particle and 125 Å pore size)</td>
<td>4.6</td>
<td>31</td>
<td>7.0</td>
</tr>
</tbody>
</table>

From this table the positive impact of method development can be seen. The retention time of the first peak was reduced from 17.8 minutes to 4.3 minutes which reduced the length of the method. The quantity of GU values that could be detected also increased from 9 GU to 33 GU. The resolution of the peaks was also improved, particularly in the high GU species. For example, in the first method, it was not possible to determine the resolution of the peak corresponding to GU 15 due to coelution. As method performance was improved, chromatographic resolution was also improved with the initial method allowing the detection of 9 GU compared to 33 GU and 31 GU when using the final methods for each column, which demonstrates the high resolution that can be achieved with an analytical (4.6 mm) and microbore column (2.0 mm).

The development of these methods has led to the production of four other methods that can be used in the analysis of biologically relevant carbohydrates with 2-AB 10, ProA 21 and its alkyne derivative 35. These methods have been successfully applied to a few different sample types (GHP, RNase B glycans and IgG glycans) and labels, but these methods were applied in varying degrees of success. The analysis of the labelled GHP samples were comparable to the analysis that is observed when this analysis is performed with HILIC. However, the labelled RNase B and IgG samples were not comparable to the equivalent HILIC analysis with HILIC giving superior specificity. The different mechanisms of separation between the two modes of HPLC resulted in coelution for the RP analysis whilst the HILIC analysis showed superior resolution and selectivity.
The measures of chromatographic performance were evaluated for each of the eight methods. Each method showed well resolved, easily discernible peaks at suitable intensity for detection. This was demonstrated by the resolutions, peak area and S/N values that were calculated for each injection of each method. Peak resolution was calculated with values all over 1.5 indicating sufficient resolution. S/N values were also all over 10 which means quantitation is possible. The repeatability of the eight methods was also all seen to be adequate from the relatively low RSD values for retention times. RP-nano-LC-MS analysis was also performed on the labelled GHP samples. This analysis illustrated the ability to confidently assign and detect the labelled glycan ions with high mass accuracy.

Although these RP methods developed in this chapter allowed the detection of glycans and could, if required, be used in the analysis of glycans, these methods have certain limitations. Retention in RP is largely determined by the label and this means retention will change significantly depending on the label employed. This could mean that each label may require a different method and therefore a large number of methods may be required in RP-HPLC to allow the use of every label. The differences in retention between different glycans may also mean that different methods are required for different sample types. This is a disadvantage compared to HILIC methods, in HILIC one HPLC method can be used to analyse a wide variety of sample types with a wide variety of labels. The HILIC analyses performed in both Chapter 3 and this chapter were all performed with the same method.

Coelution is another limitation of RP-HPLC analysis of glycans. As glycans in RP-HPLC are separated on glycan size, coelution will occur. Therefore, if detection is performed with FLD, coelution of analyte, which was observed in the RP analysis of the labelled RNase B and IgG samples but not in the HILIC analysis of the same sample, meant the total number and structure of glycans cannot be determined reliably in some cases. In HILIC with FLD detection, peaks elute based on their hydrophilicity which gives superior specificity and resolution and also means that
any coeluting analytes will have very similar structures which can still allow estimation of the glycan structures.

This coelution also means that retention in RP-HPLC does not correlate well with the elution of retention standards such as GHP, so these standards provide no reliable indication of GU and cannot be used to infer structure. An arabinose ladder may provide improved ability to infer the structure of an unknown species to a known retention standard in order to calculate GU values\textsuperscript{342}. Therefore, potential structures may be assigned more easily, and this could be investigated in future work.

To achieve structural estimation in RP-HPLC, it may require comparison of unknown peaks with labelled glycan standards. This would require a great number of standards of common glycans with all of the commonly used labels. The consistent elution order in HILIC leads to easier putative structural assignment and will likely remain the most commonly used method in glycan analysis. It is unlikely that RP-HPLC with FLD will replace HILIC-FLD in glycan analysis and explains why this analysis is rarely seen in the literature.

Where RP-HPLC does have more potential for adoption is in analysis paired with MS detection. The nature of MS means that exact masses can be determined which will allow structural determination. Coeluting peaks and inconsistent elution between the varieties of glycans or the glycan labels will not prevent structural determination. The use of MS detection would provide the ability to characterise the coeluting peaks for the labelled RNase B and IgG in this work. Glycan analysis with RP-HPLC-MS would provide greater access to this field of analysis. Laboratories experienced in RP-HPLC analysis could adopt these methods as the apparatus, such as the columns, may already be available in the lab preventing the need to purchase expensive HILIC columns. In the near future however, it is likely that HILIC will continue to be the main method of HPLC FLD analysis for these samples, limitations withstanding.
4.5 Experimental

*General experimental*

RP HPLC was performed on a Waters 2695 separating module with a Waters 474 fluorescence detector. Separation was performed on either a Phenomenex Aqua microbore column (150 mm × 2 mm, 3 μm particle and 125 Å) or a Phenomenex Aqua analytical column (75 mm × 4.6 mm, 5 μm particle and 125 Å).

HILIC was performed on an Agilent 1100 series HPLC system. Detection was performed using an Agilent 1100 series G1321A FLD detector. The column for the separation was a TSKgel® Amide-80 HR (25 cm × 4.6 mm, 5 μm particle size) with a TSK® guardgel Amide-80 (1.5cm × 3.2 mm, 5 μm particle size) guard column.

NanoLC-MS analysis was performed using an UltiMate 3000 RSLC nano liquid chromatography system (Thermo Scientific) coupled to a LTQ Orbitrap Velos mass spectrometer (Thermo Scientific) via an EasySpray ESI source (Thermo Scientific).

FLD of ProA 21 labelled glycans was performed at $\lambda_{\text{ex}}$ 299 nm and $\lambda_{\text{em}}$ 361 nm. FLD of alkyne derivative 35 labelled glycans was performed at $\lambda_{\text{ex}}$ 296 nm and $\lambda_{\text{em}}$ 361 nm. FLD of 2-AB 10 labelled glycans was performed at $\lambda_{\text{ex}}$ 320 nm and $\lambda_{\text{em}}$ 420 nm.

*Solutions and buffers*

RP-HPLC was performed with a mobile phase composed of 0.1% FA (aq) solvent A and 99.9% MeCN and 0.1% FA as solvent B and were filtered and degassed by before use.

HILIC was performed with a mobile phase composed of ammonium formate (aq) (50 mM, pH 4.4) as solvent A and solvent B consisting of MeCN, both degassed by vacuum filtration.
4.5.1 Preparation of carbohydrates

Preparation of GHP was performed using the same method as the general procedure presented in Chapter 3.5.2.1

Enzymatic deglycosylation and purification of RNase B and IgG glycans was performed by the same method as the general procedures presented in Chapter 3.5.2.4 and 3.5.2.5 respectively.

4.5.2 General carbohydrate labelling

Reductive amination of a GHP was performed with the same method as the general procedure presented in Chapter 3.5.3.1. Dried labelled samples were reconstituted in H₂O (2 mL).

Reductive amination of RNase B glycans and IgG glycans was performed with the same method as the general procedure presented in Chapter 3.5.3.2. Dried labelled samples were reconstituted in H₂O (100 µL).

4.5.3 RP-HPLC of labelled carbohydrates

4.5.3.1 RP-HPLC of ProA 21 and 35 labelled GHP and RNase B glycans.

RP-HPLC analysis of ProA 21 or alkyne derivative 35 labelled GHP or RNase B glycans was either performed with a Phenomenex Aqua analytical column (75 mm × 4.6 mm, 5 µm particle and 125 Å) or a Phenomenex Aqua microbore column (150 mm × 2 mm, 3 µm particle and 125 Å) at a column temperature of 25 °C. Analysis performed with the Phenomenex Aqua analytical column (75 mm × 4.6 mm, 5 µm particle and 125 Å) was performed with a flow rate of 1 mLmin⁻¹. Labelled GHP solution (1 µL) or labelled RNase B glycan solution (5 µL) was separated with a
gradient starting at 3% solvent B before increasing to 3.2% at 40 minutes, then increasing to 3.8% at 80 minutes. Conditioning and reequilibration was then performed by increasing solvent B to 10% by 85 minutes which was held until 90 minutes, then solvent B was decreased to 3% at 95 minutes and held until 100 minutes.

Analysis with the Phenomenex Aqua microbore column (150 mm × 2 mm, 3 μm particle and 125 Å) column was performed with a flow rate of 0.35 mLmin⁻¹. Labelled carbohydrate, either GHP solution (1 μL) or labelled RNase B glycan solution (5 μL), was separated with a gradient started at 20% solvent B before increasing to 30% at 60 minutes, then held constant at 30% until 70 minutes. Conditioning and equilibration was then performed by increasing solvent B to 100% at 75 minutes which was held until 80 minutes, then solvent B was decreased to 20% at 85 minutes and held until 90 minutes.

4.5.3.2 RP-HPLC of 2-AB labelled GHP and RNase B glycan

RP-HPLC analysis of 2-AB labelled GHP or RNase B glycans was either performed with a Phenomenex Aqua analytical column (75 mm × 4.6 mm, 5 μm particle and 125 Å) or a Phenomenex Aqua microbore column (150 mm × 2 mm, 3 μm particle and 125 Å) at a column temperature of 25 °C. Analysis with the Phenomenex Aqua analytical column (75 mm × 4.6 mm, 5 μm particle and 125 Å) was performed with a column temperature of 25 °C and a flow rate of 1 mLmin⁻¹. Labelled GHP solution (1 μL) or labelled RNase B glycan solution (5 μL) was analysed with a gradient starting at 2% solvent B before increasing to 3.5% at 41 minutes, then increasing to 4% at 70 minutes which was held until 80 minutes. Conditioning and reequilibration was then performed by increasing solvent B to 10% at 85 minutes which was held until 90 minutes, then solvent B was decreased to 3% at 95 minutes and held until 100 minutes.
Analysis with the Phenomenex Aqua microbore column (150 mm × 2 mm, 3 μm particle and 125 Å) used a flow rate of 0.35 mLmin⁻¹. 2-AB labelled GHP solution (1 μL) or RNase B glycan solution (5 μL) was injected for analysis with a gradient starting at 20% solvent B before increasing to 35% at 41 minutes, then increasing to 40% at 70 minutes. Conditioning and reequilibration was then performed by increasing solvent B to 100% at 75 minutes which was held until 80 minutes, then solvent B was decreased to 20% at 85 minutes and held until 90 minutes.

4.5.3.3 RP-HPLC of ProA 21 and 35 labelled IgG glycans

RP-HPLC analysis of ProA 21 or alkyne derivative 35 labelled IgG glycans was either performed with a Phenomenex Aqua analytical column (75 mm × 4.6 mm, 5 μm particle and 125 Å) or a Phenomenex Aqua microbore column (150 mm × 2 mm, 3 μm particle and 125 Å) at a column temperature of 25 °C. Analysis with the Phenomenex Aqua analytical column (75 mm × 4.6 mm, 5 μm particle and 125 Å) was performed at a flow rate of 1 mLmin⁻¹. Labelled IgG glycan solution (5 μL) were injected for analysis with a gradient starting at 3.5% solvent B before increasing to 5.4% at 80 minutes. Conditioning and reequilibration was then performed by increasing solvent B to 10% at 85 minutes which was held until 90 minutes, then solvent B was decreased to 3% at 95 minutes and was held until 100 minutes.

Analysis with the Phenomenex Aqua microbore column (150 mm × 2 mm, 3 μm particle and 125 Å) was performed at a flow rate 0.35 mLmin⁻¹. Labelled IgG glycan solution (5 μL) was injected for analysis with a gradient starting at 25% solvent B before increasing to 65% at 60 minutes, then increasing to 70% at 70 minutes. Conditioning and reequilibration was then performed by increasing solvent B to 100% at 75 minutes which was held until 80 minutes, then solvent B was decreased to 20% at 85 minutes and held until 90 minutes. Labelled IgG glycan solution (5 μL) was injected for analysis.
4.5.3.4 RP-HPLC of 2-AB 10 labelled IgG glycans

RP-HPLC analysis was either performed on a Phenomenex Aqua analytical column (75 mm × 4.6 mm, 5 μm particle and 125 Å) or a Phenomenex Aqua microbore column (150 mm × 2 mm, 3 μm particle and 125 Å) with a column temperature of 25 °C. The analysis performed on a Phenomenex Aqua analytical column (75 mm × 4.6 mm, 5 μm particle and 125 Å) was performed with a flow rate of 1 mLmin⁻¹. Labelled IgG glycan solution (5 µL) was injected for analysis with a gradient starting at 2% solvent B before increasing to 3.5% at 30 minutes, then increasing to 3.9% at 55 minutes and further increasing to 4.7% at 70 minutes. Solvent B was maintained until 80 minutes. Conditioning and equilibration were then performed by increasing solvent B to 10% at 85 minutes which was held until 90 minutes, then solvent B was decreased to 2% at 95 minutes and held until 100 minutes.

RP-HPLC analysis performed with the Phenomenex Aqua microbore column (150 mm × 2 mm, 3 μm particle and 125 Å) was performed with a flow rate of 0.35 mLmin⁻¹. Labelled IgG glycan solution (5 µL) was injected for analysis with a gradient starting at a gradient of 25% solvent B before increasing to 70% at 65 minutes which was maintained until 70 minutes. Conditioning and reequilibration was then performed by increasing solvent B to 100% at 75 minutes which was held until 80 minutes, then solvent B was decreased to 20% at 85 minutes and held until 90 minutes.

4.5.4 HILIC-HPLC-FLD analysis of 2-AB 10 labelled carbohydrate

HILIC-HPLC was performed via the same chromatographic method as described in 3.5.4. 2-AB 10 Labelled GHP solution (1 µL) or 2-AB 10 labelled RNase B glycan solution (5 µL) or 2-AB 10 labelled IgG glycan solution (5 µL), respectively, was injected for analysis.
4.5.5 RP-nano-LC-MS of labelled GHP

GHP samples labelled with either ProA 21 or alkyne 35 (5µg) were injected and loaded onto a trapping column (Thermo Scientific Acclaim PepMap 100 C18, 5 µm particle size, 100µm × 20 mm) for pre-concentration at 8µLmin⁻¹ in 0.1% TFA. Following valve switching the trapping column was in-line to the analytical column (Thermo Scientific Acclaim Pepmap RSLC C18, 2 µm particle size, 75µm × 50cm) and elution was carried out at a flow rate of 250 nLmin⁻¹ with 0.1% FA in H₂O as solvent A and 99.9% MeCN, 0.1% FA as solvent B. Glycans were separated with a gradient starting at 0.2% and increasing to 5% solvent B at 36 minutes. Gradient was then increase from 5 to 35% solvent B over another 14 minutes followed by subsequent column conditioning and equilibration. Eluted glycans were analysed by the mass spectrometer operating in positive polarity mode using a mass spectral resolution of 30,000 (at m/z 400). A scan range of m/z 350 – 2000 was used and spectra were recorded in profile mode.
Chapter 5 - Investigation of phenylenediamine derivatives as quantitative sialic acid labels

5.1 Background

5.1.1 Structure of sialic acids

Sialic acids are nine-carbon acidic monosaccharides which are present in both N-linked and O-linked glycans as the terminal saccharide in glycoproteins and glycosylated lipids of higher organisms 356. Eighty sialic acid species exist, these structures show high structural variation due to the possible substituents which may be present 13, 357. The groups that contribute to the structural diversity of sialic acid is shown in Figure 88.

Figure 88: The general structure of sialic acids. The R groups representing possible derivatise illustrating the diversity in the structure of this molecule.
In addition to the possible substituions detailed in Figure 88, sialic acids can be linked with galactose at R-2 and R-4, GalNAc at R-2, GlcNAc 6 at R-2, fucose at R-4, glucose 1 at R-6 and other sialic acids at R-2, R-6 and R-7. Sialic acids can also form lactones with the hydroxyl groups at the C-1 position.

5.1.2 Roles of sialic acids

In an organism sialic acid has been observed as both bound and free. Bound sialic acids are glycosidically bonded to another molecule whereas FSA are not bonded to another molecule. Both free and bound sialic acids have been implicated as biomarkers for a variety of disease states.

Inoue et al (1998) reported a positive association between concentration of free 2-keto-3-deoynonic acid (KDN) 79 (Figure 89) in ovarian adenocarcinomas to the stage of the malignancy 358. FSA was isolated from a tumour biopsy and a blood sample of the patients by ethanol precipitation and purified by SEC with a Bio-Rad AG1 column. FSA was derivatised with DMB 27 then analysed by HPLC. KDN was found in 2.6-fold higher concentrations in the ascites (fluid accumulation in the peritoneal cavity) of patients with ovarian cancer. This work concluded that KDN 79 in ovarian cancer could be used as a marker for detecting reoccurrence of the disease 358.
Wang et al (2015) investigated whether free KDN 79 was present in higher concentration in other types of cancer. This research found increased levels of free KDN 79, Neu5Ac 8 and Neu5Gc 9 in throat cancers compared to matched lymph nodes of healthy patients. Samples from biopsies and pelleted FSA of homogenized tumour samples were collected by ethanol precipitation and analysed by LC-MS/MS. Free KDN 79 and Neu5Ac 8 were 2-fold higher than the concentration of free KDN 79 and Neu5Ac 8 in healthy individuals. Neu5Gc 9 concentration in homogenised tumours samples was 5-fold higher than healthy individuals. This research concluded that free KDN 79 may be useful as a biomarker in detecting early stage cancers and of prognostic value in the degree of malignancy.

FSA have also been implicated as biomarkers for liver diseases. Gruszewska et al (2014) investigated the association with FSA and liver conditions including non-alcoholic cirrhosis, primary biliary cirrhosis, chronic nonviral hepatitis and liver cancer. The FSA concentration of patient’s blood samples was determined in a colorimetric assay using a thiobarbituric acid dye. This work found that concentration of FSA differed between liver diseases, the highest increase in FSA was encountered in toxic hepatitis with a 1.3-fold increase in total FSA compared to a healthy person without. This research also noted differences in FSA concentrations were higher in patients with alcoholic cirrhosis. This research concluded that FSA concentration of patients with liver disease resulting from
alcohol abuse was higher than healthy people. Nalgirkar et al (2019) also investigated this after believing the role of sialic acid as biomarkers for alcoholic liver diseases had not been sufficiently elucidated. This research also concluded that sialic acid could be used as a tool for diagnosis and prognosis of alcoholic liver diseases.

Urine FSA is analysed to diagnose sialic acid storage diseases such as infantile sialic acid storage disease or Salla disease by detecting the increased Neu5Ac and KDN that is a hallmark of these disorders. FSA is also analysed in the food industry. For example, sialic acid is analysed in dairy products and dairy substitutes such as formula milk. Sialic acid is present in human milk and has been shown to affect immune system and cognitive development in babies. Neu5Gc is usually absent in human milk, however bovine milk contains approximately 5% Neu5Gc whilst also containing 25% less sialic acid than human milk. As formula milk is created from bovine milk, the sialic acid content is lower and at different proportions to human milk, therefore sialic acid content is analysed to ensure formula milk is safe and to see how it should be enriched to resemble human milk. Bound sialic acid is also analysed to determine the concentration of biotherapeutic sialic acids (such as Neu5Ac and Neu5Gc), which have been released by acid hydrolysis. This is to determine that they are present in safe ranges to prevent immune responses and ensure the efficacy of the medicine.

5.1.3 Sialic Acid Derivatization

As these molecules are implicated in many processes there is clear rationale for the analysis of these species. However, the analysis of sialic acid is regarded as complicated. Difficulties are encountered due to the glycan desialylation caused by acid catalysed hydrolysis and by in-source or post-source fragmentation during MALDI. The analysis of sialylated glycans is further complicated due to the
natural negative charge that is present at the labile carboxylic proton at the 1-
position which means sialic acids are negatively charged above pH 2.6. This results
in poor ionisation in positive mode MS. This negative charge also means analysis
is affected from the formation of carboxylate salts which causes analytes to appear
as multiple peaks in MS spectra, reducing ion intensity.

The detection of these analytes can be improved by a variety of methods. When
detection is performed in MALDI-MS, detection can be improved by performing the
acquisition in negative mode as the natural negative charge of the glycan benefits
detection of the analyte. Cold matrices such as 3-AQ and para-coumaric acid may also be employed to improve detection as they display lower
propensity to induce fragmentation of the analyte than hot matrices. Reduced
fragmentation of the analyte improves detection.

Other methods that may be employed to overcome the poor ionisation resulting
from the negative charge and the instability of sialic acids is by chemical
modification. These chemical modification facilitate the analysis through the
production of a more stable derivative of sialic acid. Many sialic acid
derivatisation methods exist for both bound and FSA, the common methods are
discussed in the forthcoming sections.

5.1.3.1 Sialic acids derivatization by esterificatoin

Methyl esterification is a common method of glycan sialic acid derivatisation; the
resultant product is more resistant to laser-induced decomposition. Methylation
was first performed quantitatively with methyl iodide on the sodium salt of sialic
acid (Figure 90) but has since been developed into a one-pot method using
diazomethane in organic solvents resulting in complete esterification of FSA within
one minute.
Methylation enhances sensitivity in MS by eliminating the labile acidic proton preventing the formation of adducts, resulting in simpler spectra and increased detection sensitivity. A ten-fold increase in detection sensitivity was observed for methylated sialic acid compared to native analyte\textsuperscript{168, 379}. Methylation may instead be performed with 4-((4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinum (DMT-MM), a potent activator of methylation\textsuperscript{380, 381}. This method has the advantage of making it possible to differentiate between $\alpha$-2,3- and $\alpha$-2,6-linked sialic acid as the $\alpha$-2,6-linked sialic acid forms the methyl esters with a mass increase of 14 Da whereas the $\alpha$-2,3-linked sialic acid forms a lactone with a mass reduction of 18 Da\textsuperscript{378}. Esterification may also performed with 3-alkyl-1-aryltriazenes. These triazene alkylating agents are non-toxic, stable under acidic conditions and produce high-throughput and quantitative labelling yielding reliable ionisation and reduced adduction\textsuperscript{382, 383}. Labelling of sialic acid by this method may only be used on $N$-linked glycans previously labelled with fluorescent tags\textsuperscript{383}.

5.1.3.2 Sialic acid derivatisation by amidation

Similar to esterification, the 1-carboxyl group of sialic acids may be amidated to reduce fragmentation and reduce in-source and post-source decay in MALDI-MS\textsuperscript{168, 375}. Amidation may be performed with ammonia or $^{15}\text{NH}_4\text{Cl}$ which results in a mass increase of 0.984016 m/z or 0.013019 m/z, respectively, making this derivatisation process compatible with database searching\textsuperscript{375, 384}.
Amidation may also be performed with 2-(2-pyridilamino)ethylamine (PAEA). PAEA-amidation suppresses preferential cleavage by in-source decay, post-source decay and collision induced dissociation preventing adduct formation and enhancing ion intensity. PAEA derivatization may be used to determine sialic acid linkages in MS analysis and the analyte can be detected in HPLC-FLD or TLC-UV analysis in picomolar concentrations.

Acetohydrazide amidation is another method of amidation. In this method, glycans must first be labelled or reacted when still attached to the glycoprotein before enzymatic release, as this method is incompatible with glycans that contain a free reducing terminus because derivatization would also occur on the reducing terminus. Acetohydrazide amidation results in quantitative labelling of both the α-2,3-linked and α-2,6-linked sialic acid which prevents the formation of adducts in MS analysis resulting in a six-fold increase in signal intensity for the derivatized glycan compared to the native glycan. The MS analysis of the amide product means it possible to discern different isomers.

5.1.3.3 Sialic acid derivatization by quinoxaline formation

The final method of sialic acid derivatisation detailed in this chapter is the production of a quinoxaline derivative. This reaction is commonly performed with the label DMB (Figure 91).

![Structure of DMB 27](image)
DMB 27 derivatisation is performed in dilute acetic acid with sodium dithionite as a reducing agent to stabilise the derivative against light and oxidative decomposition \(^{389}\). Quinoxaline derivatisation is a selective reaction between the phenylenediamine moiety of 27 and the 1-carboxyl group and 2-hydroxyl group of the \(\alpha\)-keto-carboxyl group on the sialic acid (Scheme 36). Quinoxaline derivatisation only occurs on the acyclic form of sialic acid, this means quinoxaline derivatisation may only be used to analyse FSA or sialic acid released from glycoprotein which is typically performed by acid hydrolysis \(^{390-394}\). Sialic acids are in equilibrium between the ring closed (cyclic) \(\alpha\)- and \(\beta\)-form and the ring opened (acyclic) form. 99.6% of the time these molecules exist as the ring closed form, 7.5% as the \(\alpha\)-form and 92.1% existing in the \(\beta\)-form and the remaining 0.4% in the acyclic form \(^{395}\).

![Scheme 36: Neu5Ac 8 derivatisation with DMB 27.](image)

DMB 27 is a fluorogenic compound; these are compounds that are not initially fluorescent but may become fluorescence once modified by a chemical or biochemical process \(^{396, 397}\). This presents an advantage over other methods of labelling as fluorescence only occurs after formation of the quinoxaline, meaning
the reaction mixture can be analysed immediately after derivatization and any unreacted labels do not require removal before analysis.

As quinoxaline derivatisation occurs at C-1, it prevents the formation of the negative charge of the analyte \(^{168, 389}\). The formation of the products also results in an increase in hydrophobicity and introduces a fluorescent group. The derivatised product then may be analysed by HPLC-FLD or HPLC-MS. HPLC-FLD analysis yields structural information of the derivatized sialic acid when compared to labelled sialic acid standards; the analysis of the quinoxaline labelled product may be analysed in HPLC-MS for structural characterisation \(^{392}\). DMB 27 derivatisation has found large scale employment in sialic acid analysis. Klein et al (1997) analysed a variety of sialic acids released from bovine submaxillary mucins labelled with DMB 27 by LC-ESI-MS. This research identified 28 different sialic acids, bearing various modifications including 7-acetylation, 9-acetylation and 9-lactyl esterification \(^{398}\).

However, there are drawbacks to this method of derivatisation. DMB 27 is expensive and both DMB 27, and the quinoxaline labelled product are unstable in light and prone to oxidation \(^{389}\). The instability of the label presents difficulty in the handling and labelling of these samples. The labelling reaction should be performed in darkness to prevent decomposition and the derivatised product should be analysed as soon as possible after preparation.

Evidence of this instability was seen in the analysis of samples stored for 17 days at -40 °C. The stored analytes were analysed alongside freshly prepared analyte. In comparing these analyses, variation across replicates was 35% higher in the stored samples than the freshly prepared samples which was attributed to continued derivatisation of the sample \(^{399}\). Peak area, which indicates the concentration of the analyte, also changed. A 12% reduction was seen for the stored samples compared
to the freshly made samples thought to be due to the degradation of the product.

Although the instability of DMB 27 has been highlighted in the literature, few studies have examined the instability of the derivatised products. Kleikamp et al (2020) attributed the degradation of the product to DMB 27 rather than the general quinoxaline product. Kleikamp et al (2020) determined this in the analysis of nonulosonic acids, including Neu 7, Neu5Ac 8 and KDN 79 labelled with 27. These analytes were analysed by ESI-MS and two species were detected that were attributed to degradation products. The first was the result of chemical instability at the 4,5-methylenedioxy end of the label, whereas the other degradation product was produced from unknown fragmentation events.

This observation that instability is attributed to the label rather than the quinoxaline product was also stated by Wang et al (2014). This research investigated the quinoxaline derivatisation of sialic acid to verify whether 4,5-dimethylbenzene-1,2-diamine may be used as a cheaper alternative to DMB 24. The labelling of Neu5Ac 8 and Neu5Gc 9 with this molecule formed a quinoxaline product which displayed increased stability compared to the DMB 27 labelled species indicating that the label is the origin of the instability, rather than the quinoxaline product.

5.2 Aims

The work in this chapter investigates the potential for commercially available phenylenediamine derivatives in the analysis of sialic acids. These labels were hoped to provide the same selective, fast derivatization reaction that is seen with DMB 27, while also producing a quinoxaline product that could be detected in
greater sensitivity, with greater stability. The methods used and the labels employed in this section are shown in the flowchart in Figure 92.

Chapter 5

- Investigate the fluorogenicity and fluorescence parameters of commercial ortho-phenylenediamine derivatives 27, 81, 82 and 83
  - HPLC analysis of 27, 81, 82 and 83 quinoxaline derivatised Neu5Ac 8
    - Investigate the linearity of 27, 81, 82 and 83 quinoxaline derivatised Neu5Ac 8
      - Investigate the stability of 27, 81, 82 and 83 quinoxaline derivatised Neu5Ac 8

Figure 92: A flowchart showing the prospective order of research in this Chapter highlighting the methods and labels employed.
5.3 Results and discussion

5.3.1 Identification of potential novel sialic acid labels

Three commercially available OPD 81 derivatives were selected to investigate their potential as alternatives to DMB 27 in sialic acid analysis. The labels selected were OPD 81, which also forms the core of 3,4-diaminobenzoic acid 82 and 2,3-dihydro-1,4-benzodioxine-6,7-diamine (DEB) 83 (Figure 93).

Figure 93: Structures of OPD 81, 3,4-diaminobenzoic acid 82 and DEB 83.

The first label, OPD 81, was selected as it had been used previously as a fluorescent probe in the analysis of substrates forming a quinoxaline group. Zhao et al (2019) described the use of OPD 81 as a fluorogenic probe in the analysis of alkaline phosphatase activity. In this research, the reaction between ascorbic acid and OPD 81 created the fluorescent quinoxaline product which was detected by FLD 401. This label was also used in the analysis of sialic acid. Anumula (1995) demonstrated that OPD 81 could be employed for labelling of FSA by labelling commercially sourced Neu5Ac 8 and Neu5Gc 9, as well as FSA released by mild acid hydrolysis from fetuin 402. This study has highlighted the use of OPD 81 as a label for the analysis of sialic acid but did not investigate the stability of the OPD 81 labelled sample. This label was selected so the stability of this label and the quinoxaline product could be investigated.
3,4-diaminobenzoic acid 82 was selected as it was similar to a label from a patent by inventors Novotny et al (1992) 403. In this patent, a diacetic acid derivative of orthophenylenediamine was used as a fluorogenic reagent in the labelling of α-ketoacids including Neu5Ac 8 to facilitate analysis by CE-LIF. 82 was selected as it was the most similar commercially available label to the molecule used by Novotny et al (1992). The similar structures were hoped to result in similar fluorogenic properties to provide sensitive detection of the derivatised Neu5Ac 8.

The final label, DEB 83 was selected due to the structure similarity to DMB 27. For this label, the structural difference is the ethylene bridging rather than the methylene bridging between two oxygen atoms. This ethylene bridge results in a six-membered ring with bonding angles that allow greater flexibility allowing the ring to exist in more stable conformations 404. This was hoped to result in a more stable label than the five-membered ring of DMB 27. As the remaining portion of the label is the same this should hopefully result in similar fluorescence properties and retention and resolution in HPLC-FLD.

5.3.2 Reaction monitoring of 21, 81, 82 and 83 labelling of Neu5Ac 8 investigate label fluorogenicity

It was hoped that these potential labels would exhibit similar fluorescent and fluorogenic properties to DMB 27; which would facilitate analysis by HPLC-FLD without removal of free label. Should these labels not show the same degree of fluorogenicity, the formation of the quinoxaline product was hoped to result in a change in fluorescence so no overlap between the fluorescence of the reacted and unreacted species occurs, preserving the ability for HPLC-FLD analysis without prior removal of the label.
The monitoring of the labelling reaction was performed to investigate the fluorogenicity of the labels and provide an estimate of stability. To determine this, change in fluorescence caused by the formation of the nascent quinoxaline product over the course of the labelling reaction was monitored. 3D fluorescence spectroscopy was performed on the labelling reaction between an equimolar (0.08 mM) mixture of Neu5Ac 8 and label in dilute acetic acid 0.15M (aq), with sodium dithionite 0.18 mM as the reducing agent, in the dark at 50 °C. A 1:1 ratio of carbohydrate: label was used to ensure a high concentration of the quinoxaline product to provide adequate detection of the quinoxaline product. Labelling at the normal ratio of label to sugar (>20:1) may result in a product at a concentration below the LOD of the fluorimeter. Neu5Ac 8 was selected for this experiment as it is the most abundant sialic acid on mammalian glycoproteins and is normally the most abundant sialic acid released from biotherapeutics 405.

A 3D fluorescence spectroscopy scan was performed between 250 and 500 nm, increasing the excitation wavelength by 10 nm in each subsequent scan except OPD 81 which started at 230 nm. The emission of each scan was recorded between 300 nm to 600 nm. Spectroscopy was performed every hour for 4 h to monitor the change in fluorescence over the course of the labelling. The 3D fluorescence spectroscopy of the labelling reaction with DMB 27 is shown in Figure 94.
Figure 94: 3D fluorescence spectroscopy of NeuSAc 8 labelling reaction with DMB 27. (a) 0 h, (b) 4 h. Each line represents the emission at the excitation wavelength from the 3D spectroscopy. c) shows the increase in emission at the most intense wavelength from this experiment at each time point indicating the increase of the concentration of labelled species as the labelling progressed.
Each line in Figure 94 (a) and Figure 94 (b) shows the emission spectra at each respective excitation wavelength. Figure 94 (a) is the 3D spectroscopy of the DMB 27 labelling solution at 0 h, whereas Figure 94 (b) shows the 3D spectroscopy of the labelling solution at 4 h. Comparing the two spectra provides the ability to monitor the emergence of the nascent quinoxaline product and evaluate the fluorogenicity of the label. The fluorogenicity of the label could be seen through the emergence of emission at approximately 450 nm, the result of the quinoxaline product. Evidence of the instability of the label may be seen in the peak at 370 nm. In Figure 94 (a) the fluorescence spectrum 0 h, a peak at 340 nm is present at higher intensity after 4 h in Figure 94 (b) which according to Wang et al (2014) was due to the instability of the label. However, they did not disclose the reasoning behind this conclusion. Figure 94 (c) shows the change in emission over the course of the labelling at an excitation of 370 nm, the wavelength that produced the highest emission intensity. This increase in emission shows the formation of the quinoxaline product as the labelling reaction proceeded.
Figure 95: 3D fluorescence spectroscopy of NeuSAc 8 labelling reaction with OPD 81. (a) 0 h, (b) 4 h. Each line represents the emission at the excitation wavelength from the 3D spectroscopy. c) shows the increase in emission at the most intense wavelength from this experiment at each time point indicating the increase of the concentration of labelled species as the labelling progressed.
The 3D fluorescence spectrum of the Neu5Ac 5 labelling reaction with OPD 81 is shown in Figure 95. Figure 95 (a) is the 3D spectroscopy of the OPD 81 labelling solution at 0 h with Figure 95 (b) showing the 3D spectroscopy of the labelling solution at 4 h. The 3D fluorescence spectroscopy started at 200 nm as 81 was estimated to possess excitation and emission maxima at lower wavelengths to DMB 27. The emergence of the nascent quinoxaline product may be identified in the increase in the emission at 360 nm. This may indicate the formation of the product, with a change in excitation and emission wavelengths compared to the unreacted label 81. No increase in intensity corresponding to the unreacted label at 360 nm was observed which could be attributed to the instability of the label and may indicate that label 81 is more stable than DMB 27.

Figure 95 (c) shows the change in emission from the excitation at 270 nm over the course of the labelling. The increase in emission indicates the formation of the quinoxaline product as the labelling reaction proceeded. This emission was observed at low intensity possibly indicating lower detection sensitivity for OPD 81 compared to DMB 27, which will result in worse detection sensitivity of the labelled product in HPLC-FLD analysis.
The 3D fluorescence spectroscopy of 3,4-diaminobenzoic acid 82 is shown in Figure 96. Figure 96 (a) shows fluorescence of the labelling reaction at 0 h and Figure 96 (b) shows the emission of the sample after 4 h. In Figure 96 (a), the unreacted label can be seen emitting between 340 - 360 nm with excitation between 270 – 290 nm.
Comparing these two spectra, no change in emission is discernible. This may mean that the quinoxaline product possesses similar fluorescence to the unreacted label, or alternatively that no derivatisation may be occurring. To investigate whether derivatisation was occurring this labelling reaction was scaled up and monitored by TLC. The TLC of this reaction is shown in Figure 97.

Figure 97: TLC of scaled up Neu5Ac 8 derivation reaction with 3,4-diaminobenzoic acid 82. The plate was ran with a mobile phase of CHCl₃ : MeOH (4:1) and visualized by UV (254 nm). N denotes a spot of Neu5Ac 8, D represents a spot 3,4-diaminobenzoic acid 82, S is a spot of sodium hydrosulphite, CS is the co-spot and R is the spot of reaction mixture.

From this TLC in Figure 97, a new species at approximately Rf 0.4 and 0.5 can be seen in the spot of the reaction mixture indicating the presence of a new species. This new spot is likely due to the formation of the 3,4-diaminobenzoic acid quinoxaline derivative indicating that 82 is not fluorogenic and the quinoxaline product of the labelling reaction shows similar fluorescence properties.
Figure 98: 3D fluorescence spectroscopy of NeuS8Ac 8 labelling reaction with DEB 83. (a) 0 h, (b) 4 h. Each line represents the emission at the excitation wavelength from the 3D spectroscopy. c) shows the increase in emission at the most intense wavelength from this experiment at each time point indicating the increase of the concentration of labelled species as the labelling progressed.
The 3D fluorescence spectroscopy of the DEB 83 labelling reaction is shown in Figure 98. Figure 98 (a) is the 3D spectroscopy of the DEB 83 labelling solution at 0 h with Figure 98 (b) showing the 3D spectroscopy of the labelling solution at 4 h. These spectra show high similarity to the DMB 27 labelling reaction in Figure 94. The emergence of the nascent quinoxaline product in this case is seen in the new emission at approximately 450 nm. This change in fluorescence indicates the emergence of a species that is very similar to what was observed in the DMB 27 labelling, indicting that this label 83 displays similar fluorogenicity.

As was seen in the DMB 27 labelling in Figure 94, a peak is present at 370 nm in Figure 98 (a). This peak is present at higher intensity and lower wavelength at 340 nm after 4 h in Figure 98 (b), identical to Figure 94 which may possibly be indicating similar instability of this label as DMB 27.

Figure 98 (c) shows the change in the excitation at 370 nm, the wavelength that resulted in the highest emission intensity, over the course of 4 h. This emergence and increase in emission at this wavelength is attributable to the formation of the quinoxaline product, as the labelling reaction proceeded and shows the fluorogenicity of label 83.

5.3.3 Fluorescence spectroscopy of 27, 81, 82 and 83 labelled Neu5Ac 8

From the 3D fluorescence spectroscopy of the labelling reaction, the wavelengths that resulted in the highest emission could be used to approximate the $\lambda_{ex}$ and $\lambda_{em}$ for the novel labels. These values would then be used to set the detector in HPLC-FLD analysis later. In this analysis, Neu5Ac 8 was labelled under the same conditions used in the reaction monitoring to investigate fluorogenicity. The single wavelength fluorescence spectroscopy was performed of the labelled product without purification. The fluorescence spectroscopy of the DMB 27 labelled sample is shown
in Figure 99. For this label the $\lambda_{\text{ex}}$ and $\lambda_{\text{em}}$ were determined to be 374 nm and 451 nm.

Figure 99: Excitation and emission fluorescence spectroscopy of Neu5Ac 8 labelled with DMB 27.

Figure 100: Excitation and emission fluorescence spectroscopy of Neu5Ac 8 labelled with OPD 81, $\lambda_{\text{ex}} = 268$ nm and $\lambda_{\text{em}} = 362$ nm.
The fluorescence spectroscopy of OPD 81 labelled Neu5Ac 8 is shown in Figure 100. For this label the λ<sub>ex</sub> and λ<sub>em</sub> were determined to be λ<sub>ex</sub> = 268 nm and λ<sub>em</sub> = 362 nm which differ from the literature values for OPD 81 labelled sialic acid product stated by Anumula (1995) reported as λ<sub>ex</sub> = 232 nm and λ<sub>em</sub> = 420 nm. Two emitting wavelengths are observed in the fluorescence spectrum, the first at 362 nm, is likely caused by emission from the quinoxaline product, whereas the emission at approximately 550 nm is possibly caused by second order diffraction. This is caused by the use of monochromators in fluorescence spectrometry which diffract light to select the desired wavelengths. However, the angles of diffraction are not unique and when diffracting a broad range of wavelengths there is an overlap between the angles of diffraction, which leads to an artifact in the emission spectrum.

The fluorescence spectroscopy of the 3,4-diaminobenzoic acid 82 labelled sample is shown in Figure 101. For this label the λ<sub>ex</sub> and λ<sub>em</sub> were determined to be λ<sub>ex</sub> 281 nm and λ<sub>em</sub> 347 nm. These values were similar to what was recorded for the OPD 81 labelled species. The fluorescence spectroscopy of the DEB 83 labelled sample is shown in Figure 102.
Figure 102: Excitation and emission fluorescence spectroscopy of DEB 83 labelled Neu5Ac 8, $\lambda_{\text{ex}} = 374$ nm and $\lambda_{\text{em}} = 454$ nm.

From this analysis, the $\lambda_{\text{ex}}$ Max and $\lambda_{\text{em}}$ Max of the labelled DEB 83 species were determined to be $\lambda_{\text{ex}} = 374$ nm and $\lambda_{\text{em}} = 454$ nm respectively; these values are similar to DMB 27 labelled Neu5Ac 8 ($\lambda_{\text{ex}} = 374$ nm and $\lambda_{\text{em}} = 451$ nm).

5.3.4 RP-HPLC-FLD of 27, 81, 82 and 83 labelled Neu5Ac 8

Once the fluorescence parameters for the labels were determined, these wavelengths could be used to set the fluorescence detector for RP-HPLC-FLD analysis. RP-HPLC-FLD analysis of labelled Neu5Ac 8 was performed to gather information on the sensitivity and resolution of the label and whether the sample can be analysed without prior purification (if no peak for the unreacted label is observed).

Aqueous solutions of Neu5Ac 8 (9.7 µM), sialic acid label (15.5 µM) were prepared. Furthermore a solution of sodium dithionite (28.1 µM) in acetic acid and 2-mercaptopethanol in water was prepared. 50 µL each of the Neu5Ac 8, sialic acid
label solution and sodium dithionite solution were combined and labelling was performed at 50 °C for 3 h in the dark.

The first RP-HPLC injection was performed with Neu5Ac 8 labelled with DMB 27. The labelling mixture was diluted 2-fold in H2O before injection. HPLC-FLD analysis was performed on an Agilent 1100 series HPLC system, with detection performed on an Agilent 1100 series G1321A FLD detector set with the fluorescence wavelengths determined previously. Separation was performed isocratically with a mobile phase composed of MeCN 10% and FA 0.1% (aq) on a Phenomenex Aqua C18 column (150 mm × 2 mm, 3 μm particle size, 125 Å).

The FLD detection of the DMB 27 labelled Neu5Ac 8 was performed at 10 gain. The trace of the injection of the labelling solution with and without the addition of Neu5Ac 8 is shown in Figure 103.
Figure 103: RP-HPLC-FLD trace of a) DMB 27 labelled Neu5Ac 8 and b) DMB 27 labelling reaction solution without addition of Neu5Ac 8. FLD was performed with $\lambda_{\text{ex}} = 373$ nm and $\lambda_{\text{em}} = 448$ nm.

The fluorogenicity of label 27 can be observed in the HPLC trace by the absence of a peak corresponding to the unreacted label. The peak corresponding to the DMB 27 labelled Neu5Ac 8 species was observed at approximately 6 minutes at 122 LU and
is only present in Figure 103 (a). As this peak is not present in Figure 103 (b) it illustrates the fluorogenicity of this label as the fluorescence is only occurring from the labelled analyte.

The next sample injected was a sample of Neu5Ac 8 derivatised with OPD 81. The first injection of this sample was performed with the fluorescence wavelengths determined experimentally ($\lambda_{ex} = 268$ nm and $\lambda_{em} = 362$ nm). This injection (not shown) produced a trace with no peaks that were attributed to the labelled species. The sample was then analysed by RP-HPLC-FLD with the fluorescence values from the literature ($\lambda_{ex} = 232$ nm and $\lambda_{em} = 420$ nm) to investigate if this resulted in a trace with a peak attributable to the labelled species 402. This injection is shown in Figure 104.
Figure 104: RP-HPLC-FLD trace of (a) OPD 81 labelled Neu5Ac 8 and (b) OPD 81 labelling reaction solution without addition of Neu5Ac 8. FLD was performed with $\lambda_{\text{ex}} = 232$ nm and $\lambda_{\text{em}} = 420$ nm.

From the RP-HPLC-FLD traces of the labelling reaction a peak corresponding to the unreacted label is present indicating the label 81 is not fluorogenic. The 3D spectrometry of the labelling reaction for label 81 showed the emergence of a new
species at a new emission wavelength but at a low fluorescence intensity. This meant it was unclear if this was the presence of the fluorogenic quinoxaline labelling product. A peak (2 minutes) corresponding to the unreacted label is clearly present in the injection of the labelling reaction and the injection of the labelling mixture without the addition of Neu5Ac 8 in Figure 104 (a) and Figure 104 (b).

The peak corresponding to labelled Neu5Ac 8 is seen at approximately 4 minutes at 134 LU. This peak is present in lower detection sensitivity than DMB 27 as the 27 labelled Neu5Ac 8 was diluted before injection, however OPD 81 was still suitably sensitive. This trace agrees with the conclusion by of Anumula (1995) which concluded that OPD 81 was effective in the analysis of sialic acid but did suffer from intense peaks resulting from unreacted label 402.

The labelling of Neu5Ac 8 with 3,4-diaminobenzoic acid 82 was then performed. The RP-HPLC analysis of 82 labelled Neu5Ac 8 is shown in Figure 105. This sample was analysed without prior dilution.
Figure 105: RP-HPLC-FLD trace of (a) 3,4-diaminobenzoic acid 82 labelled Neu5Ac 8 and (b) 3,4-diaminobenzoic acid 82 labelling reaction solution without addition of Neu5Ac 8. FLD was performed with $\lambda_{ex}$ 281 nm and $\lambda_{em}$ 347 nm.

The RP-HPLC traces in Figure 105 clearly show a peak produced by unreacted 82 indicating that 82 is not fluorogenic. The 3D fluorescence labelling reaction showed
no new emission wavelength indicating that the product of the labelling reaction could not be detected. This can also be seen in the FLD traces of these injections. **Figure 105 (a)** shows the injection of the labelling reaction. A peak resulting from the unreacted 3,4-diaminobenzoic acid 82 at 2 minutes can be seen in such abundance that it caused an overflow in the detector. **Figure 105 (b)** shows the injection of the labelling solution without the addition of Neu5Ac 8 this same peak is also present.

A peak corresponding to labelled Neu5Ac 8 species could not be seen in the FLD trace in **Figure 105 (a)**. This may be due to coelution of labelled Neu5Ac 8 with free label as a small shoulder is seen in the peak of the unreacted label which may be the result of 82 labelled Neu5Ac 8. As this RP-HPLC method was an isocratic method the gradient could not be optimized to reduce any potential coelution that may be occurring. As previous analyses had showed superior resolution and sensitivity this use of this label was not continued.

The final Neu5Ac 8 labelling was performed with the DEB 83. This product was analysed by RP-HPLC without prior dilution with a detector gain of 10. This analysis is shown in **Figure 106**.
Figure 106: RP-HPLC-FLD trace of a) DEB 83 labelled Neu5Ac 8 and b) DEB 83 labelling reaction solution without addition Neu5Ac 8. FLD was performed with $\lambda_{ex} = 374$ nm and $\lambda_{em} = 454$ nm.

**Figure 106 (a)** is the FLD trace of the DEB 83 labelled Neu5Ac 8 while **Figure 106 (b)** shows the injection of the labelling solution without the addition of Neu5Ac 8. It was expected that this labels would produce similar analysis to DMB 27 due to the
structural similarities. From Figure 106 (a) clear similarities to the DMB 27 labelled species in Figure 103 (a) are observed. The labelled DEB 83 sample resulted in an intensity of 62, a decrease compared to DMB 27 at 122 which had been already been diluted 2-fold prior to injection. This indicates that DMB 27 is the most sensitive label of those tested, but DEB 83 showed adequate sensitivity and separation meaning it could be adopted as an alternative in this analytical workflow.

The fluorogenicity of DEB 83 was also observed to be similar to the DMB 27 labelled samples as no peak attributable to the unreacted label was present in Figure 106 (a). This observation was predicted from the 3D fluorescence spectroscopy through the emergence of emission which was similar to DMB 27, reported to be fluorogenic 407.

5.3.5 Standard calibration curve of 27, 81 and 83 labelled Neu5Ac 8

Once the detection of the labelled samples were confirmed by RP-HPLC, the linearity of these labels were then investigated. The quantification of biotherapeutic sialic acid is performed on sialic acid removed from the glycoprotein by acid hydrolysis 408, 409. Sialic acid concentrations must remain within close ranges between batches to reduce the differences in the properties of the biotherapeutic. Concentrations are strictly regulated and have to be analysed to ensure they meet requirements, meaning an effective method of sialic acid quantitation is essential 121.

Quantification in HPLC may be performed via the production of a standard calibration curve, these regression models are used to compare unknown concentration of samples for quantification 410. A high degree of linearity in this standard calibration curve means more accurate quantification can be made from
the standard calibration curve. A high degree of linearity can be determined by calculating the coefficient of determination in the regression model with a high coefficient of determination indicating a high linearity.

Once again, Neu5Ac 8 was selected to investigate linearity of labelled species as it is the most common sialic acid on glycoproteins such as biotherapeutics. Neu5Ac 8 was labelled in the same manner as previous labellings. Samples of labelled Neu5Ac 8 were prepared for each label and diluted with H2O to a dilution factor of 2, 5, 10, 50, 100 and 1000. These diluted samples were analysed by RP-HPLC in triplicate, immediately after preparation from low to high concentrations, to evaluate the linearity of the samples. The peak area of the injection of each of the diluted solutions was plotted and the coefficient of determination was calculated. Once again, DMB 27 labelled Neu5Ac 8 was first diluted by a factor of 2 before being diluted further and injected as a comparison for the other labels. The overlaid traces of the injections of DMB 27 labelled Neu5Ac 8 at all concentrations, as well as the calibration curve plotted of the peak area of each injection, is shown in Figure 107.
Figure 107: The triplicate RP-HPLC-FLD analysis of diluted DMB 27 labelled Neu5Ac 8. (a) shows the overlaid FLD traces, each line represents the scale of dilution and (b) shows the peak area of the injected diluted samples.

*Figure 107 (a)* shows the overlaid traces of the DMB 27 injection. The injections show high conformity of retention of the peak. The standard calibration curve of the peak area of the DMB 27 labelled Neu5Ac 8 solutions had a coefficient of determination of $r^2 = 0.9989$ in a log-log regression model (*Figure 107 (b))*). At lower concentrations, the conformity of the results to the regression model were the lower than at higher concentrations possibly caused by errors in the dilution.
Overall, the coefficient of determination for series of diluted DMB 27 labelled samples was deemed satisfactory.

Figure 108: The triplicate RP-HPLC-FLD analysis of diluted OPD 81 labelled Neu5Ac 8. (a) shows the overlaid FLD traces, each line represents the scale of dilution and (b) shows the peak area of the injected diluted samples.

The next sample analysed was Neu5Ac 8 labelled with OPD 81 shown in Figure 108. The overlaid traces in Figure 108 (a) show the same high degree in conformity in
retention as was seen with DMB 27 in Figure 107. The plot of peak area with the diluted solutions labelled with OPD 81 in Figure 108 (b) had a coefficient of determination of 0.9996 in a log-log regression model. This $r^2$ value is slightly higher than the DMB 27 labelled samples, indicating that this label could be used in a similarly effective manner to quantify as DMB 27.

Finally, the linearity of DEB 83 was investigated by diluted samples of Neu5Ac 8 labelled DEB 83 in the same manner as the previous two labels. The overlaid injections of these samples and a plot of the peak area for these injections is shown in Figure 109.

![Graph showing fluorescence intensity over time](image-url)
The overlaid trace of DEB 83 labelled species in Figure 109 (a) also shows high conformity in retention with all peaks eluting in the same region. In Figure 109 (b), the plot of the peak area for the diluted DEB 83 labelled samples shows a coefficient of determination of 0.9975 in a log-log regression model. This $r^2$ value, although lower than what was seen with the DMB 27 labelled samples, is still suitably high.

The coefficient of determination for all these labels demonstrates high linearity. As these novel labels show the same high linearity as DMB 27 it may mean it is possible to use labels OPD 81 and DEB 83 to quantify sialic acid in the same manner as DMB 27 currently with equivalent accuracy.

5.3.6 Investigating the stability of 27, 81 and 83 labelled Neu5Ac 8
Labels 81 and 83 showed similar resolution and linearity to DMB 27 with RP-HPLC-FLD analysis. The next experiments were performed to investigate the stability of the labels. A common issue associated with DMB 27 analysis is the poor stability of both DMB 27 and the quinoxaline product of DMB 27 labelling as these molecules are light, and oxygen sensitive. This following section investigates the stability of the commercially available labels 81 and 83 compared to DMB 27. Any novel labels that exhibit superior stability to DMB 27 may prevent a common limitation of this analysis. To evaluate whether these novel labels 81 and 83 displayed improved stability over DMB 27. Neu5Ac 8 was labelled with each label in the same conditions as previous. For each sample, labelling was performed in triplicate and each sample was analysis by RP-HPLC-FLD with three technical replicates. These samples were analysed every 24 h for 72 h to monitor if any changes in the FLD-traces were observed that presented possible evidence of these samples decomposing. To limit decomposition between injections, samples were stored at -20 °C in the dark.

The first of these HPLC analyses was performed with Neu5Ac 8 labelled with DMB 27 which would be compared to novel labels 81 and 83 as DMB 27 is known to decompose. This sample was injected to investigate how this decomposition appears as a comparison to other labels. Should the analysis of the Neu5Ac 8 labelled with novel labels show reduced decomposition it may mean these labels could be used preferentially over DMB 27. The values of peak area and retention times of the labelled Neu5Ac 8 peak for each trace are shown in Table 18.
Table 18: Comparison of peak area and retention time of DMB 27 labelled Neu5Ac 8 over 72 h. Peak area values are given to 1 decimal place. Retention time, average, standard deviation and percentage relative standard deviation are given to 2 decimal places.

<table>
<thead>
<tr>
<th>Time after labelling</th>
<th>Repeat</th>
<th>Peak Area (ng/µL)</th>
<th>Retention time (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Technical replicate</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>0 h</td>
<td>1</td>
<td>4,020.4</td>
<td>3,726.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4,068.9</td>
<td>3,778.0</td>
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<tr>
<td></td>
<td>3</td>
<td>3,820.5</td>
<td>3,395.3</td>
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<td>3,451.1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3,598.1</td>
<td>3,395.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3,515.8</td>
<td>3,424.7</td>
</tr>
<tr>
<td>48 h</td>
<td>1</td>
<td>3,731.8</td>
<td>3,673.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3,724.4</td>
<td>3,590.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3,644.5</td>
<td>3,577.4</td>
</tr>
<tr>
<td>72 h</td>
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<tr>
<td></td>
<td>3</td>
<td>3,782.8</td>
<td>3,744.6</td>
</tr>
</tbody>
</table>
The data in Table 18 show clear changes in retention and intensity of the DMB 27 labelled Neu5Ac 8 sample over time which may be attributed to the decomposition of the samples. The initial peak at 0 h shows a response identical to Figure 106 (a), but in subsequent injections retention decreased by approximately 0.4 minutes after 24 h, which may have been caused by decomposition in the samples. A slight decrease in peak area of approximately 200 is also observed. At 48 h and 72 h respectively, a further minor increase in peak area was observed with approximate increase of 200 at each time point. A greater change is observed in retention time at 48 h and 72 h, as retention decreased by approximately 0.1 minutes at 48 h and increased again at 72 h by 0.2 minutes.

This pattern in retention change and peak area change was consistent in all of the replicates, this demonstrated by the low values of %RSD at the later time points. A large change in %RSD is observed for the peak area of the samples at 0 h. This was perceived to be the result of the decomposition of the analyte and illustrated the necessity to analyse the sample immediately after labelling, as a short time after labelling (in this case 5 h) a large change in peak area was observed producing variation between injections and a higher %RSD for the injections at 0 h.

The stability of the OPD 77 labelled samples was investigated next. The Neu5Ac 8 labelling was performed in triplicate with the same method as the DMB 27 labelled samples and analysed by RP-HPLC-FLD with three technical replicates at the same time points. The values of peak area and retention time of the labelled Neu5Ac 5 peak for each trace are shown in Table 19.
Table 19: Comparison of peak area and retention time of OPD 81 labelled Neu5Ac 8 over 72 h. Peak area values are given to 1 decimal place. Retention time, average, standard deviation and percentage relative standard deviation are given to 2 decimal places.

<table>
<thead>
<tr>
<th>Time after labelling</th>
<th>Peak area</th>
<th>Retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Technical replicate</td>
<td>Technical replicate</td>
</tr>
<tr>
<td></td>
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<td>2</td>
</tr>
<tr>
<td>0 h</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>2,015.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1,967.9</td>
</tr>
<tr>
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</tr>
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<td></td>
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<td>2,593.5</td>
</tr>
<tr>
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<td>3</td>
<td>2,513.0</td>
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<tr>
<td>48 h</td>
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</tr>
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<td>3,039.4</td>
</tr>
</tbody>
</table>
The data in Table 19 again shows clear changes in retention and peak area of the OPD 81 labelled Neu5Ac 8 sample over time. However, these changes unlike in the DMB 27 labelled Neu5Ac 8 may not be entirely the result of sample decomposition. The initial peak at 0 h shows a response identical to Figure 107 (a). An increase in peak area of approximately 500 is observed, which differs to the decrease for the DMB 27 labelled samples. A possible explanation for this observation is continued labelling of the sample between injections. However, retention decreased by approximately 0.2 minutes after 24 h, which may indicate possible decomposition of the samples. At 48 h and 72 h respectively, a further increase in peak area is observed with an approximate increase of 300 at 48 h and a further 200 at 72 h. A retention decrease of approximately 0.1 minutes is also observed at 48 h before a minor increase in retention time is seen ranging from 0.03-0.06 minutes at 72 h.

Once again, this pattern in retention time and peak area change was consistent in all of the replicates, demonstrated by the low values of %RSD at the later time points. The large change in %RSD observed for the peak area of the DMB 27 labelled samples at 0 h was not as obvious here, which could indicate that the OPD 81 labelled samples are initially more stable directly after labelling than the DMB 27 labelled sample but decomposition does still occur as time passes.

Finally, the stability of the DEB 83 labelled samples was investigated. Labelling was performed in triplicate in the same method as previous, the HPLC-FLD injection of each sample was performed with three technical replicates. The values of peak area and retention times of the labelled Neu5Ac 8 peak for each trace are shown in Table 20.
Table 20: Comparison of peak area and retention time of DEB 83 labelled Neu5Ac 8 over 72 h. Peak area values are given to 1 decimal place. Retention time, average, standard deviation and percentage relative standard deviation are given to 2 decimal places.

| Time after labelling | Repeat | Peak area | | Retention time | | |
|----------------------|--------|-----------|--------|----------------|--------|
|                      |        | Technical replicate |       | Technical replicate |       |
|                      |        | 1 | 2 | 3 | AVG | STD | RSD | 1 | 2 | 3 | AVG | STD | RSD |
| 0 h                  | 1      | 1,413.0 | 1,476.0 | 1,431.17 | 39.06 | 2.73 | 9.65 | 9.67 | 9.67 | 9.66 | 0.01 | 0.12 |
|                      | 2      | 1,473.0 | 1,517.5 | 1,503.77 | 26.70 | 1.78 | 9.67 | 9.67 | 9.67 | 9.67 | 0.00 | 0.00 |
|                      | 3      | 1,474.9 | 1,535.1 | 1,507.87 | 30.51 | 2.02 | 9.66 | 9.67 | 9.68 | 9.67 | 0.00 | 0.01 |
| 24 h                 | 1      | 1,801.9 | 1,831.3 | 1,819.70 | 15.65 | 0.86 | 9.20 | 9.33 | 9.42 | 9.32 | 0.11 | 1.19 |
|                      | 2      | 1,881.8 | 1,951.4 | 1,919.67 | 35.14 | 1.83 | 9.25 | 9.33 | 9.45 | 9.34 | 0.10 | 1.08 |
|                      | 3      | 1,884.8 | 1,913.7 | 1,903.87 | 16.51 | 0.87 | 9.29 | 9.39 | 9.48 | 9.39 | 0.10 | 1.01 |
| 48 h                 | 1      | 2,042.3 | 2,039.8 | 2,045.03 | 7.01 | 0.34 | 8.94 | 9.02 | 9.03 | 9.00 | 0.05 | 0.55 |
|                      | 2      | 2,167.0 | 2,184.7 | 2,181.90 | 13.72 | 0.63 | 8.97 | 9.03 | 9.03 | 9.01 | 0.03 | 0.38 |
|                      | 3      | 2,179.1 | 2,210.2 | 2,202.27 | 20.39 | 0.93 | 9.00 | 9.04 | 9.03 | 9.02 | 0.02 | 0.23 |
| 72 h                 | 1      | 2,143.3 | 2,148.8 | 2,145.9 | 2.76 | 0.13 | 9.00 | 9.16 | 9.32 | 9.16 | 0.16 | 1.75 |
|                      | 2      | 2,383.2 | 2,377.2 | 2,378.03 | 4.80 | 0.20 | 9.07 | 9.26 | 9.38 | 9.24 | 0.16 | 1.69 |
|                      | 3      | 2,385.4 | 2,392.5 | 2,388.67 | 3.58 | 0.15 | 9.12 | 9.28 | 9.44 | 9.28 | 0.16 | 1.72 |
The data in Table 20 again shows clear changes in retention and intensity of the DEB 83 labelled Neu5Ac 8 sample over time. The change in peak area in this case is more similar to the OPD 81 labelled analyte than the DMB 27 labelled sample, possibly indicating further labelling occurring after the first injection. The initial peak at 0 h shows a response identical to Figure 109 (a), but possible indication of product decomposition may be observed in later analyses. The retention time of the labelled Neu5Ac 8 peak decreased by approximately 0.3 minutes while peak area increased by approximately 400 after 24 h. At 48 h and 72 h respectively, a further increase in peak area is observed with approximate further increase of 200-300 at 48 h and 100 at 72 h. The same pattern in changing peak retention is also observed, retention decreases by approximately 0.3 minutes at 48 h, followed by a minor increase in retention time ranging from 0.1-0.2 minutes at 72 h. This pattern in retention change and peak area change was consistent in all of the replicates demonstrated by the low %RSD values.

The changes in retention time in FLD traces of the novel labels and DMB 27 were consistent over 72 h indicating instability for each of the quinoxaline product. This analysis coupled with a colour change in the solution of label that was observed for each label over time may indicate that instability is the result of the phenylenediamine group or the quinoxaline product in general, rather than DMB 27 that had been theorised by Wang et al (2014) 389. The oxidative instability of para-phenylenediamine has been reported previously by Meyer et al (2015), resulting in the formation of a benzoquinonine diamine radical 413. The light instability of OPD 81 has also been observed via the oxidative coupling of OPD 81 producing diaminophenazine after irradiation 414. Similar processes may account for the instability of sialic acid labelling with OPD 81 derivatives.

5.4 Conclusion and future work
The wide variety of implications that sialic acids are observed to play present a clear incentive for the analysis of FSA. However due to the structure of sialic acid and the presence of the natural negative charge, sialic acid analysis is accompanied with desialylation from the glycan and poor ionisation of FSA. Sialic acid analysis using derivatisation such as DMB 27 labelling for both free and released sialic acid have provided an effective method of analysis but suffer from limitations. DMB 27 is expensive and both the label and derivatised product are unstable which limits this method of derivatisation as the handling and use of these samples are complicated. This instability was attributed to the label itself in the literature, therefore the research in this chapter aimed at finding alternatives to DMB 27 which would potentially display greater stability and greater detection sensitivity this may be adopted as an alternative to DMB 27 in sialic acid analysis.

Three potential commercially available phenylenediamine molecules 81, 82 and 83 were selected to investigate the potential of these molecules in sialic acid analysis. First the fluorogenicity of the labels were evaluated experimentally with 3D fluorescence spectroscopy of a Neu5Ac 8 labelling reaction. The fluorogenicity of DMB 27 ensures that no purification is required prior to HPLC analysis. The DEB 83 labelling reaction showed similar results to DMB 27 with the emergence of an intense emitting species after four hours. The DEB 83 labelled analyte resulted in a 9-fold increase in this emission intensity over the course of the labelling while the DMB 27 labelled analyte resulted in an 8-fold increase in detection intensity over the course of the labelling reaction indicating similar fluorogenicity for both labels. The OPD 81 monitoring showed a small change in emission after 4 h which possibly indicated a low degree of fluorogenicity, but monitoring of the 3,4-diaminobenzoic acid 82 labelling reaction showed little change in fluorescence indicating no fluorogenicity.

Next the fluorescent properties of the labelled Neu5Ac 8 were determined by fluorescent spectroscopy. The OPD 81 labelled Neu5Ac 8 had maximum fluorescent
wavelengths at $\lambda_{\text{ex}} = 268$ nm and $\lambda_{\text{em}} = 362$ nm. The 3,4-diaminobenzoic acid 82 labelled Neu5Ac 8 had maximum fluorescent wavelengths at $\lambda_{\text{ex}} = 281$ nm and $\lambda_{\text{em}} = 347$ nm, while DEB 83 labelled Neu5Ac 8 had maximum fluorescent wavelengths at $\lambda_{\text{ex}} = 374$ nm and $\lambda_{\text{em}} = 454$ nm.

These values were used to set the fluorescence detector in RP-HPLC-FLD. The injection of 3,4-diaminobenzoic acid 82 labelled Neu5Ac 8 showed no evidence of the labelled product. The analysis of Neu5Ac 8 labelled with OPD 81 when analysed with the fluorescent wavelengths determined by fluorescence spectroscopy produced a trace with no peak present corresponding to the labelled species. However, when analysed with the fluorescent wavelengths reported by Anumula (1995) a resolved peak corresponding to the labelled Neu5Ac 8 was discerned from the large peaks produced by unreacted label. The RP-HPLC analysis of Neu5Ac 8 labelled with DEB 83 also produced a trace featuring a resolved peak corresponding to the labelled analyte. Both the product of labelling with both labels OPD 81 and DEB 83 were detected with suitable resolution and showed potential in analysis of sialic acid. However, both labels were less sensitive than DMB 27 as analysis of the DMB 27 labelled sample resulted in similar detection intensity after dilution.

The RP-HPLC-FLD analysis of the labelled quinoxaline product with the isocratic method was suitable for these analytes, but it would be interesting to analyse a greater variety of sialic acid. Although Neu5Ac 8 is the most common sialic acid, sialic acids are a diverse family and it is necessary to investigate the separation and resolution of other sialic acids when labelled with either OPD 81 or DEB 83. The results of those analyses may illustrate the requirement for optimisation or altering of this HPLC method.

The linearity of the labels were then investigated in the system by RP-HPLC-FLD analysis of the labelling reaction in triplicate. Both labels 81 and 83 were plotted
and the coefficient of determination calculated. The $r^2$ value for both labels were suitably high at $r^2 = 0.9996$ for the OPD 81 labelled samples and $r^2 = 0.9975$ for DEB 83 labelled samples and were similar to the DMB 27 labelled samples at $r^2 = 0.9989$.

The investigation of stability for these novel labels was also performed. The analysis of the OPD 81 and DEB 83 labelled samples over 72 h showed changes in retention and peak area. An overall average change in retention time -0.4, -0.2 and -0.4 was observed for labels 27, 81 and 83 respectively and an overall average change in peak area at -130, 1010 and 820 was observed for labels 27, 81 and 83 respectively. The change in retention was consistent for all labels and may be attributable to decomposition of the sample indicating that the instability is not caused by DMB 27, but may be a result of the phenylenediamine group or quinoxaline product in general.

The commercially available phenylenediamines investigated in this analysis showed potential. These labels did not show any increased stability compared to DMB 27 which could introduce further applications for this analysis, such as high throughput screenings, but the instability of the quinoxaline product has not prevented the use of DMB 27. Therefore labels 81 and 83 could be adopted as cheaper alternatives in this field of analysis. However, further validation of these labels must be performed with a wider variety of sialic acids, from a variety of sources.
5.5 Experimental

General experimental

All chemicals were purchased from commercial sources (Fisher Scientific, Sigma Aldrich, Alfa Aesar, Acros Organics, Fluorochem) and used without further purification. TLCs were carried out on aluminium backed Merck Millipore TLC silica gel 60 F254 plates. Visualisation of TLC plates was accomplished by UV (λ = 254 nm).

Analytical equipment

Fluorescence spectroscopy was performed on a Varian Eclipse Fluorescence spectrophotometer. HPLC was performed on an Agilent 1100 series HPLC system. Detection was performed using an Agilent 1100 series G1321A FLD detector. Fractions were collected with an Agilent 1100 series G1364B preparative fraction collector. The column for the separation was a Phenomenex Aqua microbore column (150 mm × 2 mm, 3 μm particle size and 125 Å).
5.5.1 3D fluorescence spectroscopy of Neu5Ac 8 labelling reactions.

Sialic acid label and Neu5Ac 8 were each dissolved separately in water to a final concentration of 0.08 mM. Sodium dithionite was dissolved in water to a final concentration of 0.18 mM. 40 µL of each solution was added to 0.197M acetic acid (aq) (380 µL) to produce a solution at a final volume of 500 µL and a final concentration of 0.15M acetic acid (aq). This solution was transferred to a fused quartz fluorescent cuvette and analysed by 3D fluorescence spectroscopy with a slit of 5 nm. The 3D fluorescence spectroscopy of the labelling reaction was monitored at 50 °C, with the sample excited originally at 250 nm then every 10 nm until 500 nm, while emission was recorded between 300 nm and 600 nm.

5.5.2 General labelling of Neu5Ac 8 standard

Water (1500 µL), glacial acetic acid (172 µL) and 2-mercaptoethanol (112 µL) were mixed in a glass vial. To this solution, sodium dithionite (4.9 mg, 28.14 µmol) was added and then agitated by pipette action until dissolved. Sialic acid label (15.5 µmol) was dissolved in water (200 µL) and combined with the sodium dithionite solution.

Neu5Ac 8 (3 mg, 9.7 µmol) was dissolved in water (1 mL). 250 µL of this solution was diluted with water (24.3 mL). Neu5Ac 8 solution (50 µL) was added to label solution (50 µL). The combined solution was heated in darkness at 50 °C in an oven for 3 h. After 3 h, reaction was halted by freezing at -20 °C. Samples were injected onto HPLC as soon as possible after labelling.

5.5.3 General RP-HPLC-FLD analysis of labelled Neu5Ac 8
RP-HPLC was performed isocratically with solvent A composed of 10% MeCN and 0.1% FA (aq) for 30 minutes with a column oven at 25 °C. Sialic acid labelling reaction solutions (5 µL) were injected for each analysis. The DMB 27 labelled samples were diluted two-fold prior to injection.

5.5.4 General dilution of labelled Neu5Ac 8 standard to investigate linearity

Labelled Neu5Ac 8 solution was diluted with water to a dilution factor of 2, 5, 10, 50, 100 and 1000. The DMB 27 labelled samples were diluted two-fold prior to further dilution. These diluted samples were analysed by RP-HPLC-FLD immediately after preparation.

RP-HPLC was performed isocratically with solvent A composed of 10% MeCN and 0.1% FA (aq) for 30 minutes with a column oven at 25 °C. Labelled sialic acid solution (5 µL) was injected for each analysis.

5.5.4 Investigating the stability of labelled Neu5Ac 8

Sialic acid labelling was performed in triplicate following general procedure in 5.5.2. Samples labelled with DMB 27 were diluted two-fold prior to injection but Neu5Ac 8 labelled with other labels were injected without dilution. These samples were then analysed by RP-HPLC-FLD with the HPLC method from 5.5.3.
Chapter 6 – General discussion

The collective endeavour of the research in this thesis was to develop glycan analysis workflows. The first of these developments aimed at synthesising new multifunctional glycan labels that retain amine functionality required for labelling, but also feature an alkyne group to allow further analysis of the labelled glycan post analysis. The next aim focused on determining the ability of the multifunctional labels to act as glycan labels. This was to be evaluated by the analysis of labelled GHP, RNase B glycans and IgG glycans using HILIC and RP mode HPLC alongside the same glycans labelled with a commercially available label acting as a comparison. Then the unique point of these multifunctional labels, the ability to conjugate the alkyne group on the multifunctional glycan labels with an azide, would be investigated to determine whether it is possible to introduce further analysis of collected labelled glycans.

This thesis also aimed to find commercially available OPD derivatives which would act as a more stable and cheaper alternative to DMB 27 in the quinoxaline derivatisation of FSA. The sensitivity and stability of the FSA labelled product of the quinoxaline derivatisation with commercially available OPD derivatives would be analysed by RP-HPLC analysis and compared to the DMB 27 labelled product to determine which of the labels gave the most sensitive and stable product.

These aims and the experiments that would be employed to determine them were summarised in Figure 28 in Chapter 1. Figure 110 is recreation of this figure showing the degree of success for each of these experiment which is discussed in the following sections.
Figure 110: Recreation of the flowchart from Chapter 1 summarising the research undertaken in this thesis. The colour of the speech bubble indicate how successful each of the aims have been.
The research undertaken in Chapter 2 commences with the synthesis of novel multifunctional glycan labels 35 and 36, derivatives of currently used glycan labels with additional terminal alkyne group modification to introduce further uses to derivatised glycans but retaining the amine group for glycan linking. The final method of preparation for the multifunctional label 4-amino-N-(2-ethyl(prop-2-yn-1-yl)amino)ethyl)benzamide 35 was performed over 5 steps with an overall 60% yield. Label 36 which had previously been developed in the laboratory was prepared over 4 steps with an overall 61% yield.

The development of glycan labels 35 and 36 in this thesis present labels that could be functionalised through selective chemical reactions after HPLC analysis, but these labels may be modified further to introduce other potential benefits. The addition of an activated NHS carbamate is one possible adaption. Recent developments in this field have focused on decreasing the time of the labelling reaction. Glycan release, purification and labelling is a time consuming process with typical time scales of 2 days per sample. The labelling reaction itself typically requires between 2-4 hours. Development of the labelling reaction by employing labels that contain an activated NHS carbamate such as RF-MS 26 have decreased the length of the labelling reaction to 5 minutes. More labels are starting to feature activated carbamates which are aimed at reducing the time of the labelling reaction. These labels include 6-aminoquinoly-N-succinimidyl carbamate 84, InstantAB 85 and InstantPC 86 (Figure 111). This modification to labels 35 and 36 could be performed in an effort to reduce the length of labelling reaction and avoid the use of toxic reagents such as borohydride and cyanoborohydride, which in turn may increase the adoption of these labels in glycan analysis workflows. However, these labels only derivatise N-glycosylamine bearing N-linked glycans rather than aldehyde terminated N-linked glycans in reductive amination labelling which may limit their use.
The quantum yield of the multifunctional labels could also be evaluated to compare

the sensitivity of the label to other commonly used alternatives. Sensitivity is a

factor in the adoption of labels, the more sensitive the label the greater the ability
to detect low abundant glycans which is an important aspect of these workflows.

Figure 111: Structures of 6-aminoquinolyl-N-succinimidyl carbamate 84, InstantAB 85 and InstantPC 86.

The research in Chapter 3 successfully applied these multifunctional labels in glycan

analysis workflows. Initially the fluorescence properties of labels 35 and 36 were
determined so that they may be used as detection parameters in FLD analysis. The

wavelengths of the excitation and emission maxima of 35 labelled maltopentaose
($\lambda_{\text{ex}} = 296 \text{ nm and } \lambda_{\text{em}} = 361 \text{ nm}$) were determined by 3D fluorescence spectroscopy
while other group members determined excitation and emission maxima of 36 ($\lambda_{\text{ex}}$
$= 281 \text{ nm and } \lambda_{\text{em}} = 346 \text{ nm}$). These fluorescence wavelengths were used to
investigated the linearity and LLOD of 21 and 35 labelled maltoheptaose. The

coefficient of determination for this analysis showed high linearity for the 35
labelled maltoheptaose (0.9999) which was comparable to the 21 labelled
maltoheptaose (0.9997) control. The LLOD of 35-labelled standard (10 pmolmL$^{-1}$)
was identical to the 21 labelled maltopentaose control.

These multifunctional labels were then tried in the analysis of glycans. The analysis
of GHP showed high similarity to the control label 21 lacking the alkyne group. A
plot of RTNT against GU for the first 20 GU of the 21-, 35- and 36-labelled GHP was
produced and the coefficient of determination of these plots resulted in similar $r^2$
values for the labels 21 (0.9988), 35 (0.9995) and 36 (0.9950) illustrating that
accurate structural deductions may be made for labels 35 and 36. The analysis of
21, 35 and 36 labelled RNase B and IgG glycans likewise showed identical elution patterns and similar signal intensities and RTNT illustrating the possibility for adopting labels 35 and 36 into glycan analysis workflows 143.

The addition of the alkyne group introduced the ability to undergo the fast, robust, and water insensitive CuAAc reaction on labelled carbohydrates post separation 232. This provided the opportunity to react azide containing molecules that are hydrophobic and conjugated to introduce physical properties that benefit FLD and MS increasing the detection sensitivity of low abundance analytes that would not be detected otherwise 232, 244, 245, 419. Four azide conjugation partners were successfully synthesised. Azides 67 and 68 were successfully synthesised both in 29% yield. Azide conjugation partner 69 in 10% yield, with methylation of 69 producing 70 in 45% yield. The azide conjugation conditions were established in a reaction between 35 labelled lactose and commercially sourced azide conjugation partner 33 in screening 15 conditions. The conditions selected were 0.3 eq of copper sulfate and sodium ascorbate in MeCN : H₂O (2:1) at 40 °C for 4 h after formation of the conjugated product was confirmed by MALDI-MS.

The ability for the azide conjugation partners to improve the detection sensitivity of labelled glycans was then aimed to be confirmed by DI-MS, similar to previous research performed by Hunter-Walker et al (2011) 420. However, this research did not successfully confirm that these conjugation reactions resulted in an azide conjugated product that displayed greater detection sensitivity with FLD or MS detection compared to the unconjugated labelled glycan as had been aimed.

In the immediate future the possibility to increase detection sensitivity of low abundance through the azide conjugation of a hydrophobic and fluorescent azide conjugation partner should be established to evaluate whether this method could allow characterisation of low abundance glycans and more comprehensive glycome
identification \(^{420}\). Furthermore, the presence of the multifunctional label allows a variety of other possible applications not mentioned as yet to be investigated. The synthesis of biomolecules such as therapeutic peptides using solid phase protein synthesis is now an established routine process \(^{421, 422}\). The Nobel prize winning efforts of Merrifield mean that peptides and proteins can be synthesised with little effort by liquid handling systems \(^{423}\). In the case of challenging protein targets, native chemical ligation of synthesised peptide thioesters has been achieved successfully. However, it is not the same for glycans. Automated glycan assembly of polymers and GAGs is not a routine task. The synthesis of highly branched structures are complicated due to difficulties encountered with stereoselectivity and regioselectivity \(^{424}\). The labelling of a pool of glycans with \(^{35}\) or \(^{36}\), followed by HILIC-HPLC analysis and collection of the characterised analyte provides a potential method of obtaining highly branched and diverse glycan structures from a variety of natural sources for use in further analysis if the alternative of synthesising these glycans proves too challenging or not economical. These further analyses may include glycan immobilisation for surface modification or conjugation to biomolecules. One further potential possible application for collected labelled glycans obtained from this process is the investigation of glycan interaction using array technology. Carbohydrate arrays or glycan arrays have numerous oligosaccharides or polysaccharides immobilized on a solid support in a defined arrangement. Glycans have been immobilised on silica plates \(^{425}\), beads \(^{426}\) and ELISA plates \(^{427, 428}\) and have been used to investigate hundreds of carbohydrate protein interactions. \(^{429-431}\). The miniaturisations of the arrays, microarrays, have allowed surfaces with tens of thousands of analytes to be attached to a single microscope slide \(^{432}\). These microarrays permit high throughput investigation of protein-carbohydrate interactions. Recognition is performed via detection with either a fluorescently labelled sample \(^{425}\), MS \(^{433}\) or surface plasmon resonance \(^{434}\).

The research in Chapter 4 investigated the use of RP mode HPLC methods in the analysis of glycans, leading to the development and validation of eight methods for the RP-HPLC-FLD analysis of \(^{10, 21}\) and \(^{35}\) labelled GHP, RNase B glycans and IgG.
glycans that can be used as alternatives to HILIC. The development of the RP methods started by selecting the parameters of the method. A mobile phase of 0.1% FA (aq) was selected as phase A and MeOH 99.9% and FA 0.1% as B as these are directly compatible with MS detection. The Phenomenex Aqua® column was selected due to previous literature in this area demonstrating the requirement for a high aqueous gradient and the Phenomenex Aqua® column is stable under 100% aqueous conditions. A gradient was developed on a sample of ProA 21 labelled GHP with gradient optimisation leading to a method that effectively separated labelled GHP with satisfactory resolution for both the 35 and 21 labelled samples. A separate method for the 2-AB 10 labelled GHP was developed. This was required due to the separation on RP being significant influenced by the nature of the label. Analysis for all labels were also similar to the HILIC analysis in elution order. These two methods were also applied to the RNase B glycans labelled with 21 and 35 and 10 respectively. However, these methods were applied less successfully with three peaks observed indicating coelution had occurred.

The influence of glycan structure on RTNT that was seen in the analysis of the labelled RNase B samples meant the IgG labelled samples required a separate method to be developed. Samples of 10 and 35 labelled IgG glycans were used to optimise the gradient, thereby developing a further two methods for the analysis of IgG glycans. However, once again whilst separation was observed, not all of the 13 peaks corresponding to each of the most abundant glycans in the IgG glycoform were present indicating that coelution occurred in this analysis as well.

These four methods were validated by performing analysis in triplicate and comparing RTNT, peak area, peak height, resolution and S/N. The differences in RTNT, peak area and peak height were low as demonstrated by low RSD values, indicating high reproducibility of the methods. However, while these methods gave high reproducibility and resolution, variation was observed in some injections. Due to this, and the coelution that was observed, further optimisation was attempted.
Mobile phase B was changed to a 10% MeOH solution in water with 0.1% FA to reduce any potential errors in gradient proportioning that may have been resulting in some of the variation. Analysis was adapted to a microbore column with a smaller ID to reduce band spreading, increasing resolution and sensitivity as well as reducing coelution. The reduced ID of the microbore column made the previous methods incompatible so further optimisation of the gradient was performed.

Two new methods for the analysis labelled GHP and labelled GHP were created. Once again these resulted in well resolved traces with same elution orders to HILIC analyses. The analysis of labelled RNase B glycans with these methods however showed no reduction in coelution by changing to the microbore column or altering the gradient. The production of two methods for the analysis and labelled IgG and labelled IgG was also developed on the microbore column, however as was seen with the labelled RNase B glycans, no reduction in analyte coelution was observed. These methods were also validated by injecting samples in triplicate and comparing RTNT, peak area, peak height, resolution, and S/N. The differences in RTNT, peak area and peak height were low which was again confirmed from low RSD values indicating high reproducibility. However, while the peak height and S/N was improved for analysis performed with the microbore column, resolution was not improved compared to the analytical column.

The RP-HPLC analysis performed in Chapter 4 used FLD. This meant that only putative structural assignments of the analytes could be made. This is because the RTNT from the RP analysis of labelled GHP could not be used to infer structure as elution order was not consistent as retention is directly affected by label structure, glycan substitution and bisection. Rather than developing the gradient from a sample of labelled GHP, a labelled arabinose ladder could be used as an alternative. The labelled arabinose ladder is more typical in RP analysis and may allow the method development for a gradient where coelution is not observed.

Further
work could also look at the use of MS detection in the RP-HPLC analysis of glycans. 
The RP-nanoLC-MS analysis performed in this research made it possible to narrow 
the analyte structure to a small number of potential glycans. RP-HPLC-MS analysis 
of the labelled RNase B and IgG glycans would overcome the characterisation issues 
encountered with FLD, possibly allowing more comprehensive characterisation of 
the pool of analyte.

Chapter 5 investigated alternative OPD derivatives. The three commercially 
available OPD derivatives 81 – 83 were selected due to their structural similarities 
to DMB 27. The fluorogenicity of these labels were successfully investigated with 
3D fluorescence spectroscopy of the labelling reaction over time. 81 showed a low 
degree of fluorogenicity, 82 showed no evidence of fluorogenicity and 83 showed 
similar fluorogenicity to DMB 27. Indicating that 83 could be under RP-HPLC-FLD 
analysis without prior purification of the labelling reaction. Fluorescence 
spectroscopy was used to determine the optimal absorption and emission for labels 
81 (λ_ex = 268 nm and λ_em = 362 nm), 82 (λ_ex = 281 nm and λ_em = 347 nm) and 83 (λ_ex 
= 374 nm and λ_em = 454 nm) which were used as detection parameters in later RP-
HPLC-FLD analysis.

The RP-HPLC-FLD injection of the labelling reaction for Neu5Ac 8 with 81 and 83 
showed a clear peak in the FLD trace that was attributed to the labelled product 
indicating that these derivatives could be adopted in this workflow but as these 
peaks were present in lower intensity than the product of the DMB 27 labelling 
indicating that DMB 27 had superior sensitivity. The analysis of the Neu5Ac 8 
labelling reaction with 82 showed evidence of the labelled product by TLC but no 
peak attributable to the 82 labelled product could be discerned by RP-HPLC-FLD.

The linearity of FLD of 81, 83 and control 27 labelled Neu5Ac 8 were investigated by 
RP-HPLC-FLD. The coefficient of determinations for 81 (0.9996) and 83 (0.9975) was
successfully demonstrated to be similar to 27 (0.9989) indicating analyte quantitation may be similar between all labels. Likewise similarity was also observed when investigating the stability of the quinoxaline product labelled with 81, 83 and 27. In the RP-HPLC-FLD analysis of OPD 81 and DEB 83 labelled Neu5Ac 8, a reduction in RTNT and peak area was observed over time. An identical change was also observed in the 27 labelled Neu5Ac 8 analysis. These changes were attributed to instability of the quinoxaline product indicating that these alternatives were not more stable than DMB 27.

For the research in Chapter 5, Neu5Ac 8 was chosen as the sialic acid of choice as it is the most abundant form of sialic acid in animals. However, the sialic acid family is composed of eighty members featuring diverse structures. Further research into the viability of DMB 27 alternatives could focus on analysing a wider range of sialic acids. For example, Neu5Gc 9 is analytically relevant due to immunogenicity of Neu5Gc 9 to humans and would be a good candidate for further experimentation.
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