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Grau de Química

Treball Final de Grau

Establishment of a liquid chromatography-mass spectrometry method for the analysis of glycan biomarkers of pancreatic cancer.

Establiment d'un mètode d'anàlisi de glicans biomarcadors de càncer de pàncrees per cromatografia de líquids acoblada a l'espectrometria de masses.

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“Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth.”

Jules Verne

Quiero expresar mi más sincero agradecimiento a todas las personas que han contribuido de manera significativa en la realización de mi trabajo final de grado. Sin su apoyo, este trabajo no hubiese sido posible. Quiero destacar especialmente a:

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REPORT

IDENTIFICATION AND REFLECTION ON THE SUSTAINABLE DEVELOPMENT GOALS (SDG)

The Sustainable Development Goals (SDG) can be grouped into five wide areas, known as the 5Ps: planet, people, prosperity, peace, and partnership. Taking into account that this final degree project is related to glycans, compounds actually relevant to detect prematurely the presence of diseases such as cancer, people as well as prosperity would be the areas most impacted by this project. Cancer is a major cause of death in most parts of the world, for this reason, there is a great need to establish methods of analysis for its early diagnosis, prognosis and treatment management. People health and well-being are, in part, in hands of scientific researchers who make a great effort to find out new treatments for current diseases. As long as it was achieved, it will exist cure for such widespread illnesses and there will be fewer annual deaths, contributing to prosperity. This will signify a step further in the development as a society, but not an end to future research in the same field.

More specifically, the method developed in this work will be used as reference method for the analysis of biomarker glycoproteins in further research, which directly contributes to the aforementioned objectives.

Of the 17th goals considered on the United Nations website, this work is directly related to ensure healthy lives and promote well-being at all ages. The established method and its future perspectives could contribute to prevent population from developing a serious cancer and consequently strengthen the capacity of early detection, risk reduction and management of such a complex illness.

CONTENTS

1. SUMMARY

Glycoproteins are proteins that have oligosaccharides covalently attached to the peptide backbone. These carbohydrates are also known as glycans. Protein glycans play a significant role in various cellular processes, including cell-cell recognition, signaling, and adhesion on cell surfaces. However, glycans undergo structural changes in many diseases, such as cancer. Therefore, there is a need to establish new analytical methods that aid in identifying and quantifying glycans to detect the presence of diseases.

In the present study, a reference method for the separation and identification of labelled glycans using capillary liquid chromatography (capLC) coupled with ultraviolet (UV) and mass spectrometry (MS) detection will be developed. First, standard glycans selected as model will be derivatized with aniline (AN) and procainamide (ProA) labels. To assess their degree of derivatization, they will be analysed by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS). Finally, these labelled glycans will serve for the development of a reference analytical method by capLC-UV and capLC-MS.

This reference method will be used in the future to test the status of the chromatographic system in glycoprotein biomarker research. For instance, for the analysis of the N-glycans of human alpha-1-acid glycoprotein (hAGP) under study as a potential biomarker of pancreatic cancer.

Keywords: glycan, label, reference method, liquid chromatography, ultraviolet, mass spectrometry, biomarker, pancreatic cancer

2. RESUM

Les glicoproteïnes són proteïnes que contenen oligosacàrids units covalentment a la cadena peptídica. Aquestes cadenes de carbohidrats són conegudes també com a glicans. Els glicans juguen un paper fonamental en diversos processos cel·lulars com el reconeixement entre cèl·lules, la senyalització i l'adhesió cel·lular. No obstant, aquests poden patir canvis estructurals en malalties com el càncer. Per aquest motiu, existeix la necessitat d'establir nous mètodes analítics que ajudin a la identificació i la quantificació de glicans en mostres biològiques per així detectar la presència de malalties.

En aquest treball es desenvoluparà un mètode de referència per a la separació i la identificació de glicans derivatitzats emprant cromatografia de líquids capil·lar (capLC) amb detecció ultravioleta (UV) i acoblada a l'espectrometria de masses (MS). Primerament, uns glicans patró seleccionats com a model es derivatitzaran amb anilina (AN) i procainamida (ProA). Per tal d'avaluar el seu grau de derivatització, s'analitzaran per espectrometria de masses de desorció/ionització mitjançant làser assistida per matriu (MALDI-MS). Finalment, els glicans derivatitzats serviran per desenvolupar un mètode analític de referència per capLC-UV i capLC-MS.

Aquest mètode serà utilitzat en un futur per verificar l'estat del sistema cromatogràfic en l'anàlisi dels glicans de glicoproteïnes biomarcadores, com per exemple, l'alfa-1-glicoproteïna àcida humana (hAGP), glicoproteïna descrita a la literatura com a potencial biomarcador de càncer de pàncrees.

Paraules clau: glicà, agent derivatitzant, mètode de referencia, cromatografia de líquids, ultravioleta, espectrometria de masses, biomarcador, càncer de pàncrees

3. INTRODUCTION

3.1. GLYCOPROTEINS

Glycoproteins are proteins that have oligosaccharides covalently bonded to the peptide backbone (1). These carbohydrate chains, named as glycans, can be attached to different amino acid residues in the protein chain, such as asparagine, serine and threonine. This covalent bond is due to a posttranslational process involved in proteins, well-known as glycosylation (2). Glycoproteins are abundantly found in living organisms, and they play essential roles in various biological processes like cell signaling, immune response, and cell adhesion. Protein glycosylation have a potential impact on the structure and function of proteins, affecting protein folding, solubility, stability, as well as modulating protein-protein interactions (1,3,4).

Since glycoproteins might contain more than one glycosylation site and different glycan structures can be attached to these sites, a single glycoprotein is composed by a heterogeneous mixture of multiple proteoforms (named *glycoforms*) (5).

3.2. GLYCANS

Glycans are complex carbohydrates composed of several monosaccharides, which are linked by interglycosidic bonds (4). Table 1 shows the main monosaccharides that can be found in protein glycans, along with their corresponding structure, abbreviation, and standardized symbol nomenclature proposed by the Consortium for Functional Glycomics (CFG) (6).

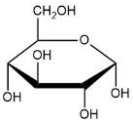
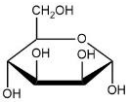
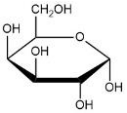
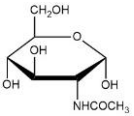
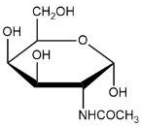
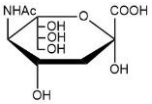
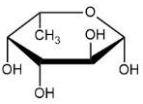
Monosaccharide	Abbreviation	Structure	Symbol
Glucose	Glc		
Mannose	Man		
Galactose	Gal		
N-Acetylglucosamine	GlcNAc		
N-Acetylgalactosamine	GalNAc		
N-Acetylneuraminic acid (Sialic Acid)	NeuAc, SiA		
Fucose	Fuc		

Table 1: Most common monosaccharides found in glycoproteins. Symbol as well as nomenclature follow the rules of the Consortium for Functional Glycomics (CFG) (6)

Two types of glycans can be distinguished depending on their attachment to the peptide backbone:

- O-glycans: they are commonly linked by an O-glycosidic bond between the hydroxyl group of a serine (Ser) or threonine (Thr) residue of the polypeptide chain and the N-acetylgalactosamine (GalNAc) at the end of the glycan (7).
- N-glycans: they are typically linked by an N-glycosidic bond between the nitrogen atom of an asparagine (Asn) side chain and the N-acetylglucosamine (GlcNAc) when it takes part of the Asn-X-Ser/Thr sequence in which X denotes any amino acid except for prolina (Pro) (7). All of them share the same central structure (core) formed by five monosaccharides (three Man and two GlcNAc units, see Figure 1), to which different monosaccharide residues are joined resulting in ramifications or *antennas* that increase their complexity and diversity. Based on their structures, N-glycans can be classified into three main subgroups: high mannose, hybrid and complex.

Since human glycoproteins such as human alpha-1-acid glycoprotein (hAGP) only contains complex type N-glycans and this work is directly related to make future glycan analysis of this protein, a brief explanation of the structural features of complex type N-glycans is provided below.

Complex N-glycans consist of a central pentasaccharide core and multiple branches known as *antennas*. These *antennas* are composed of units of N-acetyl-lactosamine (LacNAc), which comprises N-acetyl-glucosamine and galactose. Moreover, glycans frequently present a terminal sialic acid (see Figure 1) and fucoses in the core or in the antenna.

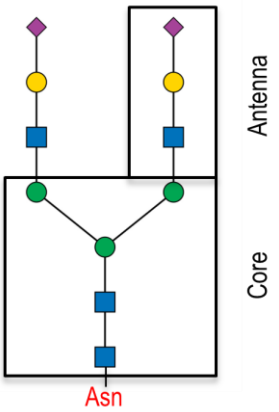


Figure 1: Basic structure of complex N-glycan. Structure has been represented using GlycoGlyph

The different linkage type of the terminal sialic acids (SiA linkage α-2,3 or α-2,6) or the fucoses (Fuc core or Fuc antenna) leads to different glycan isomers with identical atomic composition and mass (i.e. isobaric isomers). The presence of glycan isomers increases the microheterogeneity of the *glycoforms* and thus the complexity of glycoproteins.

3.3. GLYCOPROTEINS AS BIOMARKERS

Protein glycosylation plays a role in every aspect of human growth and development. Human physiological conditions can be negatively impacted by defective glycosylation, which is associated with a variety of chronic and infectious diseases. Moreover, at the beginning or during the progression of a tumor, glycosylation can become altered. Hence, glycoproteins can be excellent biomarkers that can detect cancer at early stages and distinguish it from other diseases such as chronic inflammation (8).

In particular, pancreatic cancer is one of the most aggressive cancers as well as one with a greater diagnostic difficulty. For all stages of cancer, the five-year survival rate after diagnosis is roughly 5%, but it increases to 20% for localized stages, and drops to less than 1% for patients diagnosed with advanced disease (9). The majority of pancreatic tumors are classified as pancreatic ductal adenocarcinoma (PDAC). The carbohydrate antigen 19-9 (CA 19-9) is a glycoprotein antigen released by tumor cells and it is considered, up to now, the most widely used and best-validated biomarker for PDAC. Nevertheless, the use of CA 19-9 in the early diagnosis of pancreatic cancer is limited due to its lack sensitivity and specificity, being found at high levels in PDAC but also in other pathologies such as chronic pancreatitis (10,11). Hence, future research on pancreatic cancer should prioritize the identification of biomarkers that can provide crucial information for diagnosis, prognosis and treatment selection. Additionally, they must have a high sensitivity and specificity for the disease in particular.

Human alpha-1-acid glycoprotein (hAGP), also named as orosomucoid, has a molecular weight ranging from 41 to 43 kDa. Approximately 45% of the total weight of hAGP is composed of carbohydrates. The protein is comprised of a single polypeptide chain consisting of 183 amino acids. The carbohydrate part contains bi-, tri- and tetra-antennary complex type N-glycans. hAGP has five glycosylation sites, which enables the formation of numerous *glycoforms*. However, only 12-20 of them can be detected in human serum (12).

Human α1-acid-glycoprotein (hAGP) glycosylation has been described to be altered in pancreatic cancer (PDAC) and chronic pancreatitis (ChrP) (13,14). However, it is not clear yet whether some specific glycan structures in hAGP can be used as biomarker to differentiate between these two pathologies.

3.4. METHODS OF ANALYSIS OF GLYCOPROTEINS

The analysis and characterization of protein glycosylation can be carried out at three distinct levels: intact glycoprotein, glycopeptides and glycans. Since glycan chains are altered in many prevalent human diseases such as cancer, they have high potential to serve as a target in the diagnosis of these illnesses (15). In most cases, glycans undergo enzymatic digestion with glycosidases to be released from the protein backbone. Free glycans do not show inherent chromophore or fluorophore properties and they do not ionize efficiently by mass spectrometry (MS). Furthermore, protein glycans can show several isobaric isomers, making their separation and detection a challenging task. In order to facilitate their separation and detection, and to enhance the sensitivity of the analysis, they are frequently derivatized (16,17). Numerous glycan derivatization strategies have been described in the literature. Among them, the most common methods include reductive amination (17–20), permethylation (18–21), Michael addition labelling (20) and hydrazide labelling (20).

In reductive amination, a label containing a primary amine group undergoes a condensation reaction with the aldehyde group of the glycan, forming an imine or Schiff base (see Figure 2). This intermediate is then reduced by a reducing agent to produce a secondary amine (20,22).

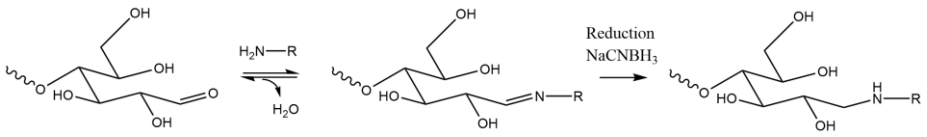


Figure 2: Reductive amination scheme between a glycan and a labelled primary amine

This labelling strategy is advantageous, since reductive amination presents a stoichiometric attachment of one label per glycan, apart from being faster and easier. Multiple labels have been used for the reductive amination of glycans. Among them, 2-aminobenzamide (2-AB), 2-

aminobenzoic acid (2-AA), 2-aminopyridine (PA) and procainamide (ProA) are typically used (18,23). All of them contain a chromophore or fluorophore group. 2-AB as well as PA lacks negative charges and they are extensively used in liquid chromatography (LC). The 2-AA carries one negative charge what makes it useful for using it in LC or capillary electrophoresis (CE) separations as well as in positive-mode and negative-mode MS analysis (20). ProA is a fluorescent label that also contains a basic tertiary amine with high proton affinity. Therefore, ProA is ionized efficiently showing high sensitivity in positive-mode MS (17,24,25). More recently, derivatizing agents isotopically labelled with stable isotopes such as ^{13}C, ^{15}N, ^{18}O and ^{2}H have also represented a very viable strategy to improve glycan quantitation by MS by relative quantitation (1,26,27).

3.4.1. MASS SPECTROMETRY

Mass spectrometry (MS) is nowadays an excellent tool for glycan analysis. Its high sensitivity and unequivocal identification of the compounds make this detection technique an ideal choice (28). Matrix-assisted laser desorption/ionization and electrospray ionization mass spectrometry (MALDI-MS and ESI-MS, respectively) have been reported in the literature for glycan analysis without using previously an electrophoretic or chromatographic separation (1,20,29). Nevertheless, the determination of minor glycans as well as the presence of glycans with sialic acids often require a separation technique prior to MS detection (1).

3.4.2. CHROMATOGRAPHIC SEPARATION

Reversed-phase liquid chromatography (RPLC) is commonly employed for separating peptides and proteins, relying on hydrophobic interactions to drive retention. However, due to the lack of interaction between hydrophilic glycans and this type of stationary phase, it becomes challenging to separate glycans by RPLC. An alternative approach is hydrophilic interaction chromatography (HILIC), where glycans perfectly interact with the stationary phase, enabling the differentiation of isomeric glycans based on variations in linkage position and/or branching (30–32).

Numerous investigations have been conducted to explore glycan separation capabilities of different HILIC columns, including the ZIC-HILIC, BEH amide and the HALO® Penta-HILIC columns (14,30,31,33–35).

The ZIC-HILIC column contains a zwitterionic group covalently attached to the silica, the BEH amide column is based on an amide group also attached to the silica, and the Penta-HILIC stationary phase is a highly polar ligand that includes five hydroxyl groups bonded to the silica as well. Their corresponding structures are depicted in Table 2.

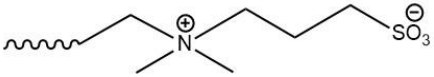
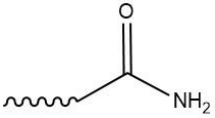
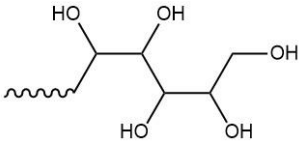
Stationary phase	Structure
ZIC-HILIC	
BEH amide	
HALO® Penta-HILIC	

Table 2: Structure of most important HILIC stationary phases for the analysis of glycans

In previous studies, our research group developed several analytical methods by capillary liquid chromatography-mass spectrometry (capLC-MS) with a ZIC-HILIC column to separate and characterize complex type N-glycans labelled with aniline (AN) of several glycoproteins of biomedical interest such as hAGP and transferrin (13,14,36,37). Despite the good results previously obtained with this column, it is currently not providing the expected results, which results in a lack of reproducibility between injections and distortion of peaks in successive analysis. For this reason, HALO® Penta-HILIC column has been selected in this study to evaluate its performance for glycan analysis and improve the results obtained by capZIC-HILIC-MS.

In this regard, with the aim of establishing a reference method for the separation and identification of glycans labelled with AN but also ProA, a set of four oligosaccharides with

different chemical nature and number of monosaccharide units has been selected in this work as model glycans: maltohexaose, LS-Tetrasaccharide a (LSTa)/ Sialyl-lacto-N-tetraose a, maltononaose and maltopentadecaose. Table 3 shows these oligosaccharides, their corresponding structures, and acronyms.

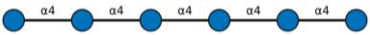
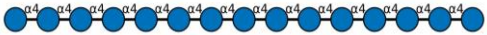
Model glycan	Acronym	Structure
Maltohexaose	MH	
LS-Tetrasaccharide a (LSTa)/ Sialyl-lacto-N-tetraose a	ST	
Maltononaose	MN	
Maltopentadecaose	DP-15	

Table 3: Model glycans used throughout this work along with their structures and acronyms. Structure has been represented using GlycoGlyph

The first step will involve their analysis using MALDI-MS, a technique known for its rapid analysis capabilities. Subsequently, these model glycans will be separated and detected by capLC-UV and capLC-MS with HALO® Penta-HILIC column. After optimization of the methods, they will be used as column test in future studies involving complex type N-glycans of glycoproteins with biomedical interest such as hAGP.

4. OBJECTIVES

The principal aim of this work is to establish a reference method for the separation and identification of labelled model glycans using capillary liquid chromatography (capLC) coupled with ultraviolet (UV) and mass spectrometry (MS) detection. These model glycans will be maltohexaose, LS-Tetrasaccharide a (LSTa)/ Sialyl-lacto-N-tetraose a, maltononaose and maltopentadecaose. Firstly, they will be derivatized with aniline (AN) and procainamide (ProA) to improve the sensitivity of the analysis. Labelled glycans will be analysed by matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) to assess their degree of derivatization. Finally, separation and detection of these model glycans will be optimized by capLC-UV and capLC-MS, and retention parameters will be determined.

5. EXPERIMENTAL SECTION

5.1. INSTRUMENTATION, REAGENTS, MATERIAL, SOLVENTS, SOLUTIONS AND SAMPLES

5.1.1. INSTRUMENTATION

- Mass spectrometer MALDI-TOF (4800 Plus MALDI TOF/TOF; ABSciex-2010) equipped with solid state laser (Nd: YAG; 355 nm) and a TOF analyser. The software used for processing data is 4800 Series Explorer.
- SpeedVac Concentrator Savant SPD 111V (Thermo Scientific)
- Centrifuge Mikro 220R (Hettich Zentrifugen)
- Centrifuge Mikro 20 (Hettich Zentrifugen)
- Ultrasonic bath Fisherbrand (FB15051)
- Thermo-Shaker TS-100 (BioSan)
- Vortex LBX instruments, V05 series
- Capillary liquid chromatography system (capLC) with an ultraviolet detector (UV), Agilent Technologies 1200 Series. The software used for processing data is LC ChemStation (Agilent Technologies).
- Mass spectrometer with quadrupole time of flight (6546 LC/Q-TOF, Agilent Technologies) with an electrospray ionization (ESI) source. The software used for processing data is Agilent MassHunter Qualitative Analysis.

5.1.2. REAGENTS AND MATERIAL

- Formic acid (Merck, 98-100% (p/p), HFor)
- Ammonia (Merck, 25% (p/v), NH_3)
- Sodium cyanoborohydride (Sigma-Aldrich, $NaBH_3CN$)

- Procainamide hydrochloride (Sigma-Aldrich, ProA · HCl)
- Aniline (Sigma-Aldrich, AN)
- Dimethyl sulfoxide (Merck, DMSO)
- Acetic acid glacial (Merck, 100% (p/p), HAc)
- Acetone HPLC/MS grade (Merck)
- 2,5-dihydroxybenzoic acid (Sigma-Aldrich, DHB)
- 2,4,6-trihydroxyacetophenone monohydrate (Sigma-Aldrich, THAP)
- α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, CHCA)
- Trifluoroacetic acid (Sigma-Aldrich, TFA)
- Diammonium hydrogen citrate (Merck)
- Centrifugal filters of polyvinylidene fluoride (PVDF) of 0.22 µm (Merck Millipore)

5.1.3. SOLVENTS

- Water, MS grade (Honeywell, H_2O)
- Ethanol, MS grade (PanReac 100% (p/p), EtOH)
- Acetonitrile, MS grade (Honeywell, ACN)
- 2-propanol, MS grade (Honeywell, iPrOH)
- Methanol, MS grade (Honeywell, MeOH)

5.1.4. SOLUTIONS

- Aqueous mobile phase: 20 mM ammonium formate pH 4.4 for capLC-MS analysis, and 50 mM ammonium formate pH 4.4 for capLC-UV analysis.

- Organic mobile phase: Acetonitrile: H_2O 95:5 (v/v).

- Glycan labelling solutions (see section 5.2.1. for detailed information)

- MALDI matrices: DHB 10 mg/mL in H_2O:EtOH (1:1), THAP 25 mg/mL in MeOH with 50 mg/mL of diammonium hydrogen citrate and 0.1% of TFA, and CHCA 10 mg/mL in TFA:H_2O:ACN (0.1:49.9:50).

5.1.5. SAMPLES

- Maltohexaose (Elicityl-OligoTech, 90% purity, MH)
- LS-Tetrasaccharide a (LSTa)/ Sialyl-lacto-N-tetraose a (Elicityl-OligoTech, 90 % purity, ST)
- Maltononaose (Elicityl-OligoTech, 90 % purity, MN)
- Maltopentadecaose (Elicityl-OligoTech, 90 % purity, DP-15)

5.2. SAMPLE TREATMENT

5.2.1. GLYCAN DERIVATIZATION

MH, ST, MN and DP-15 (10 nmol) were derivatized using different derivatizing agents:

- Aniline labelling. 10 µL of a solution containing 0.35 M of aniline and 1 M of $NaBH_3CN$ in DMSO with 30% of HAc are added to the dried model glycan samples and heated in the thermo-shaker at 70ºC for 2 hours.
- Procainamide labelling. 10 µL of a solution containing 0.25 M of procainamide and 1 M of $NaBH_3CN$ in DMSO with 30% of HAc are added to the dried model glycan samples and heated in the thermos-shaker at 70ºC for 2 hours.

Subsequently, after cooling all samples at room temperature, glycan precipitation and removal of excess reagents were performed by adding 200 µL of cold acetone (4ºC). The mixture was then vortexed and centrifuged at 14000 rpm and 25ºC for 3 minutes. To prevent glycan loss in the precipitate, the supernatant was partially removed, and this process was repeated three more times. Finally, the excess acetone was evaporated using a SpeedVac, and the dried glycans were stored at -20°C until needed.

5.3. MALDI-MS

The MALDI-TOF system was used in positive reflector mode. A total of 1500 laser shots were performed within the range of 100-3000 m/z to obtain the spectra, using a laser intensity varying from 4000 to 7900. The samples are deposited on a metallic plate with different insertion points called spots. Spots were prepared by adding 0.8 µL of the sample-matrix solution and drying them in air at room temperature.

For the preparation of the sample-matrix solution, first, model glycans (10 nmol) were dissolved in water at a concentration of 200 pmol/µL. Then, 2 µL of this solution was mixed with 2 µL of the matrix solution (described in section 5.1.4.).

5.4. CAPLC-UV

Chromatographic separation was performed using a HALO® Penta-HILIC column with a particle size of 2.7 µm, a pore diameter of 90 Å, a length of 150 mm and an internal diameter of 0.3 mm. The established experimental conditions for model glycans include a concentration of each labelled glycan in the mixture of 50 pmol/µL in water:ACN (1:1), and an injection volume of 0.25 µL. Eluting solvents were A: 50 mM of ammonium formate adjusted to pH 4.4 and B: ACN:H_2O (95:5). A detection wavelength of 245 nm and 300 nm were used for aniline and procainamide labelled glycans, respectively. Separation was performed using the elution gradient shown in Table 4. Before starting work, the two chromatographic channels used were purged for 5 minutes at 2500 µL/min and the column was conditioned at 80% of B (starting percentage of B of the gradient) at 4 µL/min for 30 min. After its use, the column was cleaned with 100% of B (ACN:H_2O 95:5) for 1 hour. The rest of the chromatographic system was cleaned with H_2O-MS for 30 minutes and then with ACN:H_2O 95:5 for another 30 minutes. If the system is not going to be used in the next two days, the capLC instrument is finally cleaned with iPrOH to prevent capillary obstructions.

Time (min)	%B
0	80
10	80
50	40
55	0
70	0
75	80
90	80

Table 4: Optimized gradient for the reference method established by capLC-UV and capLC-MS.

5.5. CAPLC-MS

Chromatographic separation was performed with the same column described in section 5.4. The established experimental conditions for the model glycans include a concentration of each labelled glycan in the mixture of 50 pmol/µL in water:ACN (1:1), and an injection volume of 0.10 µL. Eluting solvents were A: 20 mM of ammonium formate adjusted to pH 4.4 and B: ACN:H_2O

(95:5). Separation was performed using the elution gradient shown in Table 4. Aniline labelled glycans were analysed in negative ion mode (ESI-) whereas procainamide labelled glycans were in positive ion mode (ESI+). The mass spectrometer parameters used in both negative and positive modes were the same: a drying gas temperature of 200°C, a nebulizer gas flow rate of 4 L/min, a nebulizer gas pressure of 15 psig (N_2), a fragmentor voltage of 190 V, a skimmer voltage of 70 V and a radiofrequency of octopole at 300 Vpp. The only parameter that differed was the capillary voltage, which was set at 3500 V for negative mode and 4000 V for positive mode. Spectra were acquired continuously at a rate of 1 spectrum per second, covering the m/z range from 100 to 3200 at high resolution (4 GHz).

6. RESULTS AND DISCUSSION.

Many methods for the analysis of oligosaccharides do require a glycan derivatization step. The reason is that glycans are often quite complex and heterogeneous molecules, and chemical derivatization can enhance their detectability after electrophoretic or chromatographic separation. Modifying one or more functional groups on the oligosaccharide molecule, typically using a reagent with chromophore or fluorophore groups, can improve chromatographic separation, increase sensitivity and selectivity by MS, by ultraviolet or fluorescence detection (20). In this study, the derivatizing agents used were aniline (AN) and procainamide (ProA), two agents previously used by the research group (38,39) and reported in the literature (20,40).

6.1. GLYCAN ANALYSIS BY MALDI-MS

A suitable tool for analysing labelled glycans as well as free glycans is matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-MS), which easily provides the mass charge ratio of the compounds of interest in a short analysis time (29,41).

In this study, model glycans (MH, ST, MN and DP-15) were analysed by MALDI-MS in positive reflector mode as free glycans and derivatized with AN and ProA. This allowed their characterization and assessment of which derivatizing agent provides better detection. 2,5-dihydroxybenzoic acid (DHB), which was previously used for glycan analysis by several authors and our research group (29,38,39,42,43), was used as MALDI matrix at different laser intensities (5000, 6000 and 7000) to evaluate the response of these four model glycans by MALDI-MS. Free MH, ST and MN showed higher peak intensity with a laser intensity of 5000 while DP-15 needed a laser intensity of 6000 (data not shown). Since DP-15 is a larger glycan (composed by 15 monosaccharides), it needed higher laser intensity for being ionized. Moreover, most of them showed $[M+Na]^+$ as the most abundant adduct, except for free ST that was $[M+H]^+$ (data not shown).

Figure 3 depicts the mass spectra of MH, ST, MN and DP-15 labelled with AN and ProA. The behaviour of them towards the laser intensity was exactly the same as the previously obtained for the free glycans. However, for all of them, the sodiated adduct ion was the most abundant. What can also be observed in Figure 3 is the tendency of peak intensity to decrease as the number of monosaccharide units increases, what is actually coherent since the bigger the glycan, the more difficult is to ionize it, with the exception of ST-ProA that showed lower intensity than MN-ProA due to the presence of the negative charge of the sialic acid. Depending on the glycan, the peak intensity of the glycan labelled with AN is either higher or lower than the glycan labelled with ProA. For example, MN and DP-15 showed lower peak intensity when they were derivatized with AN than with ProA whereas with MH and ST occurred just the opposite. Interestingly, in the mass spectra of DP-15 appeared the adduct with sodium, but also other peaks corresponding to m/z values of maltotetradecaose (DP-14), maltotridecaose (DP-13), maltododecaose (DP-12) and maltoundecaose (DP-11). Nevertheless, further experiments must be performed to be able to know if they come from the fragmentation of DP-15 or if the standard provided by the manufacturer contains these compounds as impurities. In case it was the first cause, the derivatization is not the responsible of such fragmentation because these peaks were observed with both labels and also in the free DP-15 mass spectra (see Figure 4a).

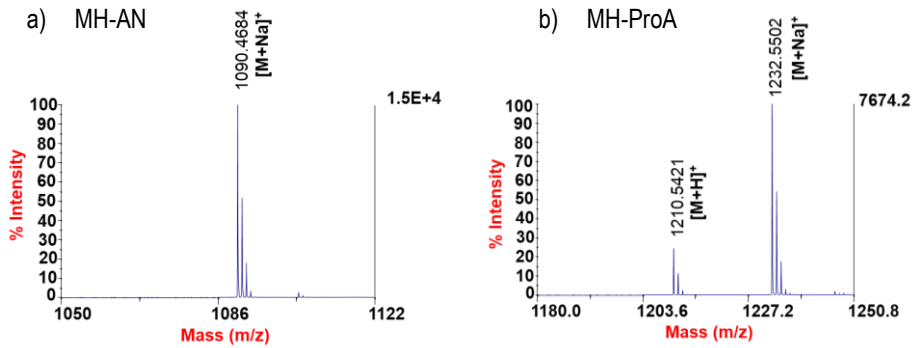


Figure 3: MALDI-MS spectra in positive reflector mode of the four labelled glycans: a) MH-AN, b) MH-ProA, c) ST-AN, d) ST-ProA, e) MN-AN, f) MN-ProA, g) DP-15-AN and h) DP-15-ProA. g) and h) obtained at a laser intensity of 6000 while the rest at 5000 (continue in the next page).

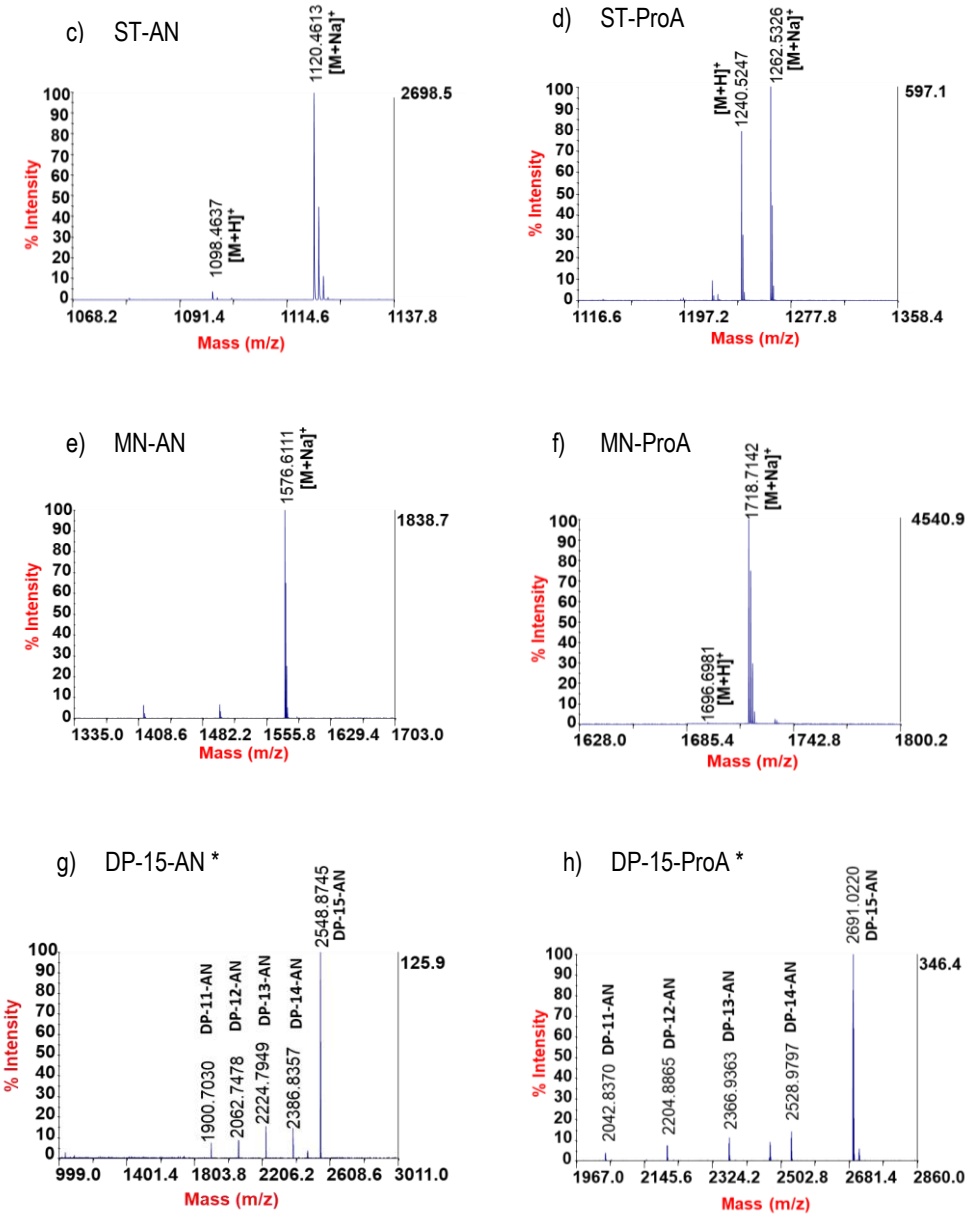


Figure 3: MALDI-MS spectra in positive reflector mode of the four labelled glycans: a) MH-AN, b) MH-ProA, c) ST-AN, d) ST-ProA, e) MN-AN, f) MN-ProA, g) DP-15-AN and h) DP-15-ProA. g) and h) obtained at a laser intensity of 6000 while the rest at 5000.

* The selected peaks in DP-15 correspond to the adduct $[M+Na]^+$

Table 5 displays the arithmetic mean of the mass charge ratio (m/z) and peak intensity, along with their corresponding relative standard deviation (RSD %), for three MALDI spots of the same sample for the four model glycans in their free form as well as labelled with AN and ProA.

Glycan	Free				Glycan-AN				Glycan-ProA			
	m/z	RSD (%)	Peak intensity	RSD (%)	m/z	RSD (%)	Peak intensity	RSD (%)	m/z	RSD (%)	Peak intensity	RSD (%)
MH	1013.40037	0.0016	8030	65.4	1090.4637	0.00078	9704.8	47.7	1232.5463	0.0003	5693.2	35.4
ST	1021.38847	0.00039	1100	67.5	1120.4514	0.0011	1951.4	33.3	1262.5383	0.0004	404.9	41.2
MN	1499.568	0.00038	1280	68.3	1576.6148	0.0002	1512.1	24.9	1718.7102	0.0003	2137.8	100.4
DP-15	2471.90023	0.00026	395	67.5	2548.8745*	-	125.9	-	2691.0151	0.0006	227.5	60.3

Table 5: Arithmetic mean of mass charge ratio and peak intensity, along with their corresponding RSD (%), made from three spots using the same sample of the four glycans, free and labelled. The peak intensity for all glycans corresponds to the adduct $[M+Na]^+$, except for free ST, which corresponds to the adduct $[M+H]^+$.

* DP-15-AN just shows signal in one spot

As can be observed in Table 5, the majority of labelled glycans presented a higher peak intensity than the free ones. However, there are some labelled glycans, specially those labelled with procainamide, that showed the opposite behaviour, what can be owing to the poor crystallization of the spot. MALDI-MS is a rapid qualitative technique; however, one of its limitations is the lack of reproducibility between spots. This is because the crystallization process can influence the resulting peak intensity. In this regard, it can be explained that DP-15-AN only showed signal in one of three spots. Moreover, if the sample was not dry enough, the aniline derivatization would be affected. Comparing between the two derivatizing agents, we can observe that AN conferred to MH and ST a higher peak intensity than that seen with ProA. At the same time, ProA gave a greater peak intensity to the larger glycans, MN and DP-15. Regarding the RSD obtained for the peak intensities, free glycans generally showed worse values than the labelled ones. This allows us to conclude that labelled glycan spots presented better reproducibility between them and that derivatization becomes a support in their analysis.

6.1.1. STUDY OF DIFFERENT MALDI-MS MATRICES FOR THE ANALYSIS OF DP-15

As described in section 6.1., the MALDI mass spectra of DP-15 showed peaks corresponding to oligosaccharides with lower number of glucoses (DP-14, DP-13, DP-12 and DP-11). In this work, several experiments were performed to investigate whether these peaks came from sample impurities or they were product of DP-15 fragmentation. First, in order to discard that the MALDI matrix itself was the responsible of such fragmentation, several matrices considered as soft and hot matrices were tested for the analysis of DP-15: DHB and two other commonly used matrices for glycan analysis by MALDI-MS (i.e. THAP and CHCA) (44).

Figure 4 displays the mass spectra of free DP-15 using DHB, THAP, and CHCA matrices. Each sample-matrix was subjected to several laser intensities to find out the optimal one that allowed us to obtain the best peak intensity. In this case, for DHB, THAP and CHCA was 6000, 7000 and 5000 respectively. CHCA required the lowest laser intensity as it is considered a hot matrix, followed by DHB with an intermediate laser intensity, and finally THAP, which required the highest laser intensity for glycan ionization.

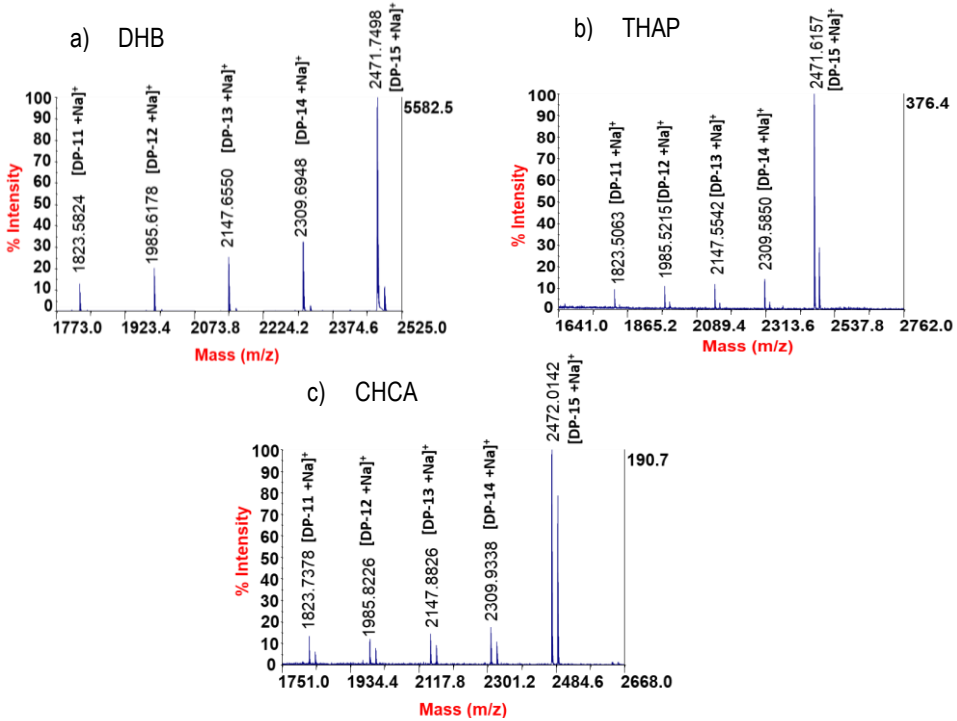


Figure 4: MALDI-MS spectra of free DP-15 in a) DHB, b) THAP, c) CHCA matrices. Laser intensity used was 6000, 7000 and 5000, respectively (positive reflector mode).

The obtained mass spectra revealed that the MALDI matrix was not the cause of DP-15 fragmentation since the possible fragments were present with all the evaluated matrices.

Finally, as the intensity of the laser was also described to promote fragmentation of labile compounds such as glycoproteins (44), we investigated whether laser intensity could be the cause of the possible fragmentation of DP-15. Several laser intensities were tested for DP-15 analysis using DHB (5000, 6000 and 7000), THAP (6000, 7000 and 7900) and CHCA (4000, 5000 and 6000). Figure 5 shows a bar graph which represents the variation in peak intensity of one of the possible fragments (DP-14) compared to the peak intensity of DP-15 using the three matrices at the different laser intensity values.

The peak intensity ratio between DP-14 and DP-15 was calculated taking the mean of three spots and the standard deviation obtained is depicted as error bars in each case.

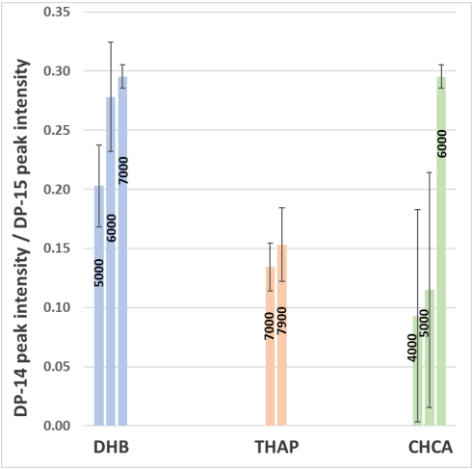


Figure 5: Evaluation of DP-14 peak intensity-to-DP-15 peak intensity ratio at different laser intensity and with different MALDI matrices (DHB, THAP and CHCA). Study carried out with three spots of the same sample-matrix.

As depicted in Figure 5, the error bars reveal that there was no significant difference observed in the DP-14/DP-15 ratio across different laser intensities, which indicates that laser intensity does not have an influence on fragmentation of DP-15. The consistent behaviour observed across all matrices further supports this conclusion. Based on the experiments carried out, we could conclude that the presence of these lower mass peaks was neither caused by the MALDI matrix nor the laser intensity.

6.2. ESTABLISHMENT OF AN ANALYSIS METHOD FOR LABELLED GLYCANS BY CAPLC-UV

Once we characterized the model glycans by MALDI-MS, this study was focused on the establishment of a reference capLC-UV method for the analysis of labelled glycans, with the purpose of being used as column test, and also to verify the proper performance of the chromatographic system in future analysis of biomarker protein glycans. The reference method was initially optimized for the analysis of aniline labelled glycans, followed by optimization for procainamide labelled glycans.

6.2.1. ANILINE LABELLED GLYCANS

- **Optimization**

Initially, aniline labelled glycans (MH-AN, ST-AN, MN-AN and DP-15-AN) were analysed by capLC-UV using a mobile phase recommended by the manufacturer of the HALO® column (31,45) (50 mM of ammonium formate at pH 4.4 in water; ACN:H_2O 95:5 (v/v)). For UV detection, the wavelength chosen was 260 nm as it was described in the literature as the optimal wavelength for aniline labelled glycans (19). AN-glycans were injected individually, at a glycan concentration of 100 pmol/µL and with an injection volume of 0.25 µL, to determine their elution time (data not shown). The gradient used for these preliminary experiments was similar to the one published by our research group for capZIC-HILIC (1,14).

Subsequently, to optimize the separation method, three different elution gradients were used for the separation of a glycan mixture by capLC-UV (glycan concentration of 50 pmol/µL to prevent column overload). Table 6 provides a visual representation of the three gradients employed for the glycan mixture separation, including the initial gradient applied to each individual glycan (gradient 1). Figure 6 shows the chromatograms obtained with these three elution gradients.

Gradient 1

Time (min)	%B
0	80
40	40
45	0
60	0
65	80
80	80

Gradient 2

Time (min)	%B
0	80
20	80
60	40
65	0
80	0
85	80
100	80

Gradient 3

Time (min)	%B
0	80
10	80
50	40
55	0
70	0
75	80
90	80

Table 6: Three gradients used for the analysis of model glycans by capLC-UV. %B corresponds to the percentage of organic solvent, in this case ACN:H_2O 95:5 (v/v).

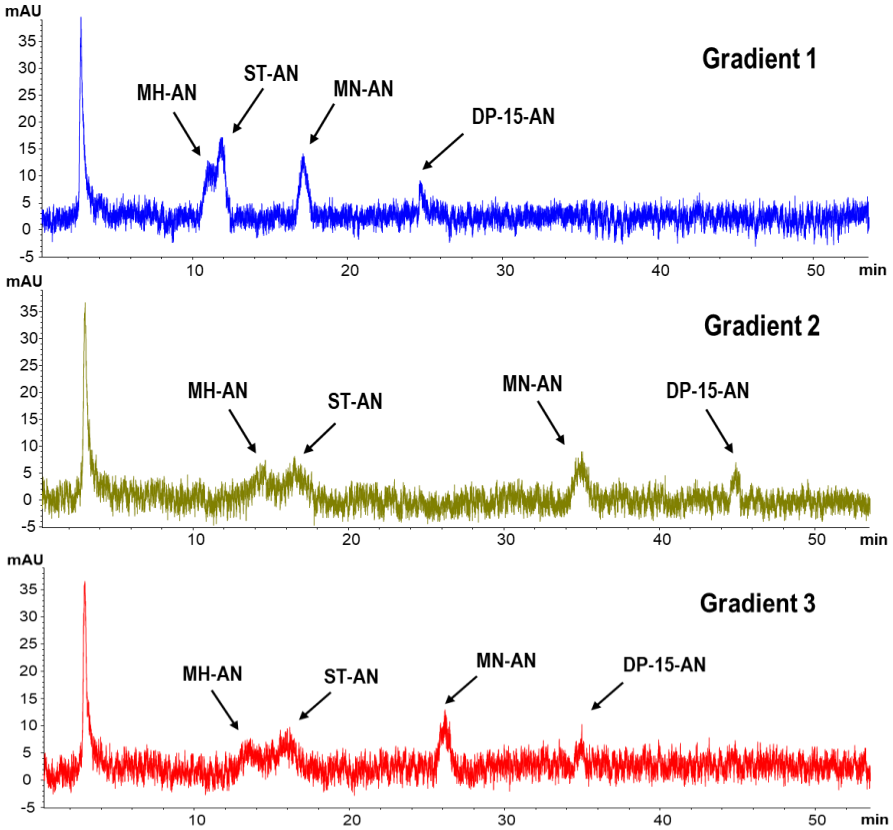


Figure 6: Chromatograms of aniline labelled glycan mixture at a concentration of 50 pmol/µL using three gradients exposed in Table 6.

Based on the observations from Figure 6, it is evident that gradient 1 failed to achieve the separation between MH-AN and ST-AN, while gradients 2 and 3 showed potential for achieving the desired separation. However, gradient 2 resulted in longer analysis times compared to gradient 3. Therefore, gradient 3 was the most appropriate and the one selected to continue our work.

As can be observed in Figure 6, the signal obtained for aniline labelled glycans was poor. To enhance detection by UV, the absorption spectrum was acquired from 230 to 360 nm, confirming that the maximum absorption wavelength corresponded to 245 nm (see Figure 7), contrary to the findings described by Xia B et al. (19). Therefore, after changing the lamp, the glycan mixture was again analysed by capLC-UV at 245 and 260 nm. Figure 8 displays the chromatograms obtained.

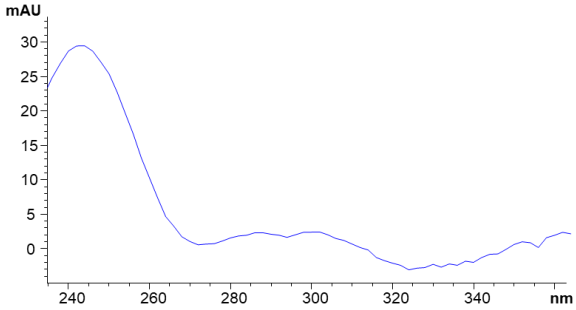


Figure 7: Absorption spectrum of MN-AN peak recorded simultaneously with the chromatogram using the definitive gradient (gradient 3 in Table 6). MN was chosen since it was well-resolved and exhibited sufficient intensity.

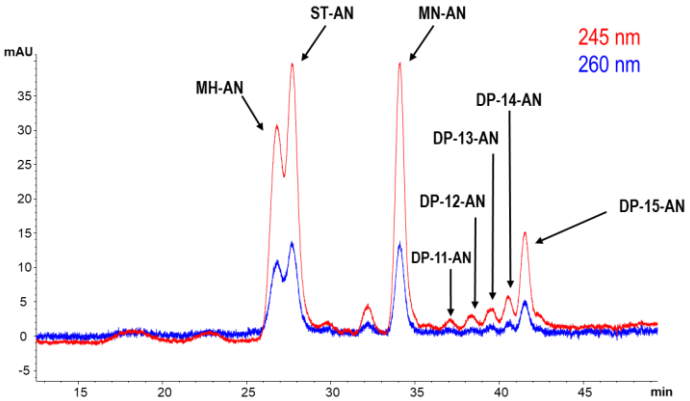


Figure 8: Chromatogram of aniline labelled glycans at 245 nm and 260 nm.

As can be seen, signal-to-noise ratio (S/N) at 260 nm was superior to that observed before (compare with Figure 6) due to a lamp change. Moreover, there was a clear difference in peak intensities between 245 and 260 nm. Consequently, 245 nm was selected as the optimal wavelength for analysing aniline labelled glycans by capLC-UV. This selection is based on the potential for achieving higher intensity at this wavelength, which can enhance the sensitivity and accuracy of the analysis.

This increase in sensitivity allowed to detect additional peaks at retention times lower than DP-15 which were due to the presence of DP-14, DP-13, DP-12 and DP-11 in the commercial pattern of DP-15 as suspected in the analysis by MALDI-MS, and finally verified by capLC-MS (see later in section 6.3). These results clearly confirmed the presence of impurities in DP-15 standard. Their retention time, peak area and relative abundance are shown in Table 7. Although the manufacturer certified 90 % purity of the standard, the relative abundance confirmed that the real purity of DP-15 was around 61% and not 90%.

	Rt (min)	Area	Relative abundance *
DP-11	37.0	58.3	6.6%
DP-12	38.2	65.8	7.5%
DP-13	39.5	75.9	8.7%
DP-14	40.5	140.4	16.0%
DP-15	41.5	536.7	61.2%

Table 7: Retention time and peak area of DP-11, DP-12, DP-13, DP-14 and DP-15 obtained from the chromatogram of Figure 8 at 245 nm. * Relative abundance was calculated as the peak area of the compound relative to the sum of the areas of all compounds.

Nevertheless, despite the impurity presence in DP-15, this model glycan is of great value to assess the correct separation of HALO® Penta-HILIC column. Thus, it was continued to be used throughout this work.

- **Retention parameters**

After selecting the optimal gradient and wavelength, retention parameters were calculated using data provided by the capLC-UV chromatogram. These parameters include retention time (Rt), peak area, theoretical plate number relative to the length of the column(N/meter), and resolution between peaks (see Table 8). The N/meter provides a direct measure of column efficiency and the resolution indicates the separation between adjacent peaks.

Glycan	Rt (min)	Area	N/meter
MH	26.8	3336.6	36035
ST	27.7	3336.6	38477
MN	34.1	1542.7	327538
DP-15	41.5	536.7	488922

	Resolution
MH and ST	0.6
ST and MN	6.2
MN and DP-15	12.1

Table 8: Retention parameters determined from the chromatogram of Figure 8 at 245 nm. N/meters refers to theoretical plate number (N) relative to the length of the column in meters (0.15 meters).

As can be observed in Table 8, DP-15 showed the highest N/meter among the other model glycans what suggests that it exhibits the smallest band broadening. However, it was the peak with the lowest intensity and the smallest area. For that reason, N/meter value of MN, which was well-resolved and showed higher signal intensity, represents more accurately the real column efficiency.

The resolution between MH and ST suggests that they were not completely baseline resolved since the resolution values between peaks in the chromatogram were below 1.5, unlike ST and MN or MN and DP-15.

6.2.2. PROCAINAMIDE LABELLED GLYCANS

- **Optimization**

As carried out with the AN-glycans, procainamide labelled glycans were injected separately in order to determine the elution time for each individual glycan at 50 pmol/µL and using gradient 1 as starting point (data not shown). In this case, the wavelength selected was 300 nm as it was described in the literature as the optimal wavelength for procainamide labelled glycans (45).

Subsequently, to optimize the separation method for ProA-glycans, gradient 1 and 3 were evaluated for the separation of a glycan mixture by capLC-UV (each glycan at a concentration of 50pmol/µL).

Figure 9 shows the capLC-UV chromatograms obtained for the mixture of ProA model glycans.

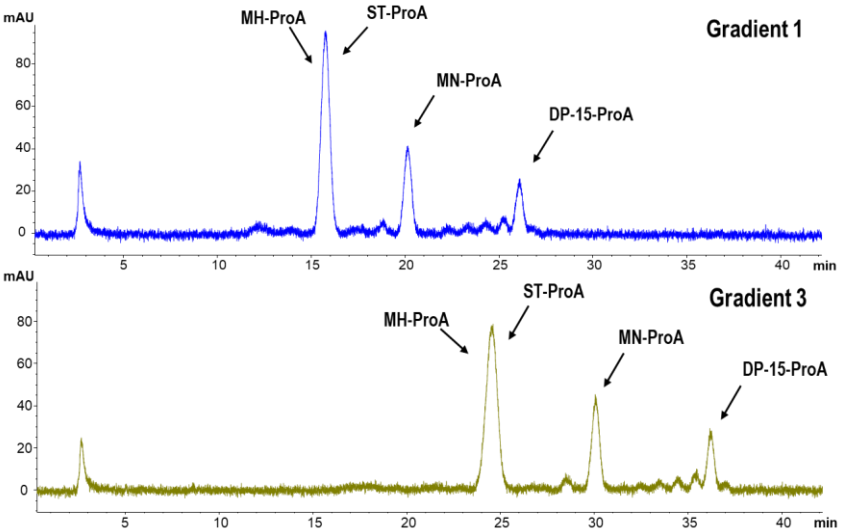


Figure 9: Chromatograms of procainamide labelled glycan mixture at a concentration of 50 pmol/µL using gradient 1 and 3 exposed in Table 6.

Based on the observations from Figure 9, neither gradient 1 nor gradient 3 successfully separated MH-ProA and ST-ProA. Other gradients with longer periods of time at isocratic conditions were evaluated but none of them provided satisfactory results. Therefore, despite having longer analysis times, gradient 3 was selected to continue our study in order to have a single reference method for the separation of both aniline and procainamide labelled glycans.

To verify the optimal wavelength of ProA-glycans the absorption spectrum was acquired from 230 to 360 nm, confirming that the maximum absorption wavelength corresponds to 300 nm (see Figure 10), as described in the literature (45).

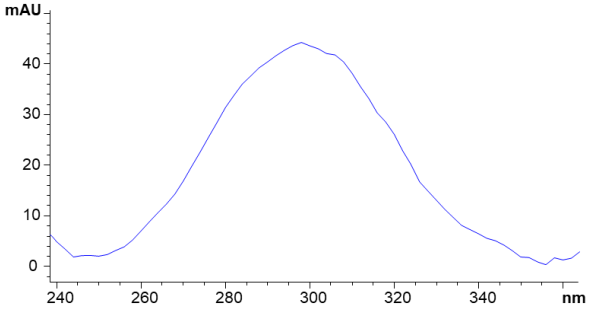


Figure 10: Absorption spectrum of MN-ProA peak recorded simultaneously with the chromatogram using the definitive gradient (gradient 3 in Table 6). MN was chosen since it was well-resolved and exhibited sufficient intensity.

- **Retention parameters**

Retention parameters were also determined as for AN-glycans labelled. These parameters include retention time (Rt), peak area, theoretical plate number relative to the length of the column (N/meter), and resolution between peaks (see Table 9).

Glycan	Rt (min)	Area	N/meter
MH	24.6	3267.3	217104
ST	24.6	3267.3	217104
MN	30.1	1216.1	578919
DP-15	36.2	608.2	1339328

	Resolution
MH and ST	0
ST and MN	11.6
MN and DP-15	16.8

Table 9: Retention parameters determined from the chromatogram of Figure 9, gradient 3. N/meters refers to theoretical plate number (N) relative to the length of the column in meters (0.15 meters).

As can be observed in Table 9, MH and ST presented the same retention time and the area integrated contained both peaks. A resolution value of zero between MH and ST suggests that these compounds were not resolved since they eluted at the same retention time value. The rest of glycans were baseline resolved, showing resolution values higher than 1.5.

These parameters obtained for AN-glycans and ProA-glycans will be taken as reference values for the HALO® Penta-HILIC column test in future analysis with this column.

6.3. ESTABLISHMENT OF AN ANALYSIS METHOD FOR LABELLED GLYCANS BY CAPLC-MS

After establishing the capLC-UV method, the scope of this work also included the development of a reference capLC-MS method for analysing labelled glycans. This method aimed to serve as a column test and also ensure the appropriate performance of the chromatographic system in future investigations of biomarker protein glycans. Initially, the reference method was optimized for analysing glycans labelled with aniline, followed by the optimization of glycans labelled with procainamide.

6.3.1. ANILINE LABELLED GLYCANS

- **Optimization**

Initially, aniline labelled glycans (MH-AN, ST-AN, MN-AN and DP-15-AN) were analysed by capLC-MS in negative ion mode (ESI-) using the mobile phase optimised by capLC-UV (50 mM of ammonium formate at pH 4.4 in water; ACN:H_2O 95:5 (v/v)). The glycan mixture was injected at a glycan concentration of 50 pmol/µL, and with an injection volume of 0.10 µL to avoid the saturation of the detector. The gradient used for these experiments was the one selected for capLC-UV method. Figure 11a shows the extracted ion chromatogram (EIC) obtained. The EIC verified that DP-14, DP-13, DP-12 and DP-11 were present in the commercial pattern of DP-15.

Subsequently, the effect of the ammonium formate content was assessed. Several lower salt concentrations (20 mM and 10 mM) were analysed (see Figure 11b and 11c) since a decrease in the ionic strength results in an increase in ionization efficiency, which positively affects sensitivity (46). As can be observed in Figure 11, 50 mM of ammonium formate showed lower intensity compared to 20 mM or 10 mM. Nevertheless, 10 mM provided broader peaks, specially for MH-AN and ST-AN. Therefore, 20 mM of ammonium formate was selected as offered better sensitivity, efficiency, and adequate peak separation.

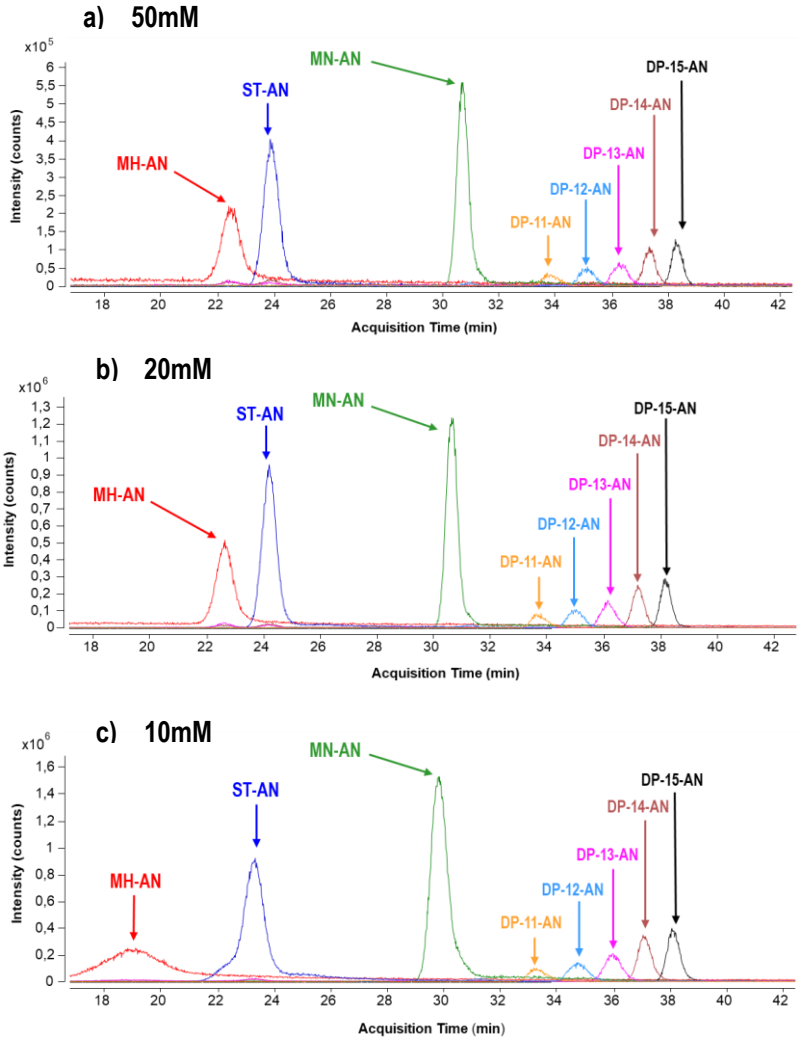


Figure 11: Extracted ion chromatograms (EICs) of aniline labelled glycan mixture at a concentration of 50 pmol/µL and using an aqueous mobile phase of ammonium formate at pH 4.4 and at different concentrations: a) 50 mM, b) 20 mM and c) 10 mM.

Finally, with the objective of improving glycan separation and detection, 20 mM of ammonium acetate at pH 4.4 was also evaluated as aqueous mobile phase because it previously provided excellent results by capZIC-HILIC-MS (14). Figure 12 shows the EIC obtained.

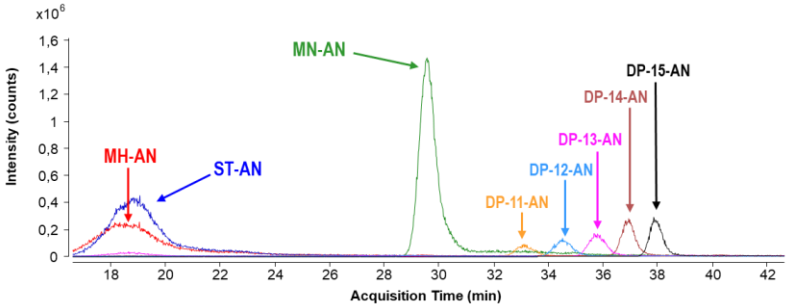


Figure 12: Extracted ion chromatogram (EIC) of aniline labelled glycan mixture at a concentration of 50 pmol/µL and using an aqueous mobile phase of 20 mM of ammonium acetate at pH 4.4.

As can be observed, ammonium acetate did not achieve separation between MH-AN and ST-AN as both glycans coeluted and peaks were broader. Thus, 20 mM of ammonium formate at pH 4.4 was the aqueous mobile phase most appropriate and the one selected to continue our work. By way of an example, Figure 13 shows the mass spectrum of MN-AN obtained with the optimised method. The observed adducts proved the presence of multicharged ions due to the ESI source.

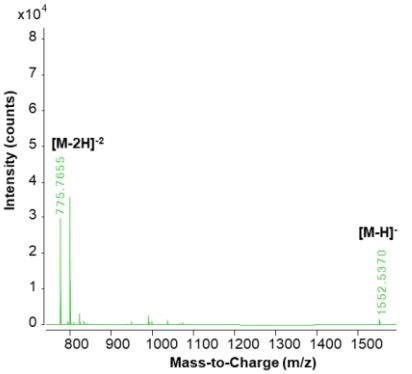


Figure 13: Mass spectrum of MN-AN obtained from the EIC of Figure 11b (negative ion mode).

- **Retention parameters**

After selecting the optimal salt type and its concentration, retention parameters were calculated using data provided by the capLC-MS chromatogram. These parameters include retention time (Rt), peak area, theoretical plate number relative to the length of the column(N/meter), and resolution between peaks (see Table 10). The N/meter provides a direct measure of column efficiency and the resolution indicates the separation between adjacent peaks.

Glycan	Rt (min)	Area	N/meter
MH	22.6	20084244	16931
ST	24.2	31781309	39067
MN	30.6	36967766	34378
DP-15	38.2	7984314	86508

	Resolution
MH and ST	1.0
ST and MN	4.3
MN and DP-15	4.9

Table 10: Retention parameters determined from the chromatogram of Figure 11b. N/meters refers to theoretical plate number (N) relative to the length of the column in meters (0.15 meters).

As observed in Table 10, the resolution between MH and ST suggests that they were not completely baseline resolved since the resolution values between peaks in the chromatogram were below 1.5. However, they were better resolved by capLC-MS than capLC-UV as they showed higher resolution by the former. The rest of glycans were baseline resolved, showing resolution values higher than 1.5.

6.3.2. PROCAINAMIDE LABELLED GLYCANS

Procainamide labelled glycans (MH-ProA, ST-ProA, MN-ProA and DP-15-ProA) were analysed by capLC-MS in positive ion mode (ESI+) using the mobile phase already optimised for AN-glycans by capLC-MS (20 mM of ammonium formate at pH 4.4 in water; ACN:H_2O 95:5 (v/v)). The glycan mixture was injected at a glycan concentration of 50 pmol/µL, and with an injection volume of 0.10 µL. The gradient used for these experiments was the one selected for capLC-UV method. Figure 14 shows the extracted ion chromatogram (EIC) obtained.

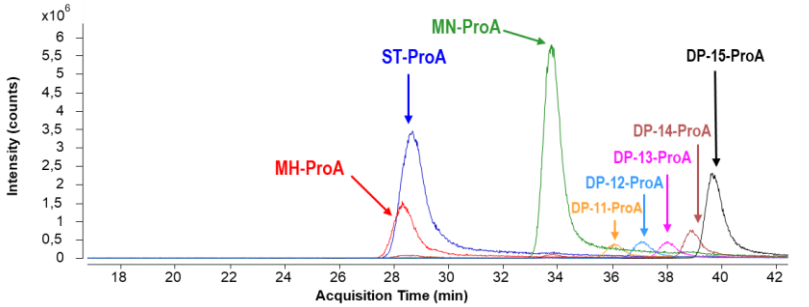


Figure 14: Extracted ion chromatogram (EIC) of procainamide labelled glycan mixture at a concentration of 50 pmol/µL and using an aqueous mobile phase of 20 mM of ammonium formate at pH 4.4.

As can be seen, as happened with capLC-UV, it was not possible to separate MH-ProA and ST-ProA. In the future, it would be necessary to evaluate different conditions such as varying pH values of the aqueous mobile phase in order to achieve this separation. By way of an example, Figure 15 shows the mass spectrum of MN-ProA obtained with the established method. The observed adducts proved the presence of multicharged ions due to the ESI source, and a superior amount of adducts compared to MN-AN in negative ion mode (see Figure 13).

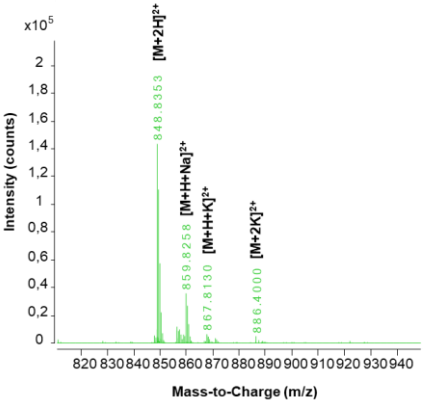


Figure 15: Mass spectrum of MN-ProA obtained from the EIC of Figure 14 (positive ion mode).

- **Retention parameters**

Retention parameters were also determined as for AN-glycans labelled. These parameters include retention time (Rt), peak area, theoretical plate number relative to the length of the column (N/meter), and resolution between peaks (see Table 11).

Glycan	Rt (min)	Area	N/meter
MH	28.3	85160163	9338
ST	28.7	204950825	7144
MN	33.8	271105534	20090
DP-15	39.7	105413745	34510

	Resolution
MH and ST	0.1
ST and MN	1.7
MN and DP-15	2.5

Table 11: Retention parameters determined from the chromatogram of Figure 14. N/meters refers to theoretical plate number (N) relative to the length of the column in meters (0.15 meters).

As can be observed in Table 11, MH and ST presented practically the same retention time. A resolution value of almost zero between MH and ST suggests that these compounds were not resolved since they eluted at the same retention time value. The rest of glycans were baseline resolved, showing resolution values higher than 1.5.

The methods established in this work using the selected model glycans by capLC-UV and capLC-MS will be employed as a column test to verify the proper performance of the chromatographic system. However, to fully achieve the separation of MH-ProA and ST-ProA by capLC-UV and capLC-MS, more experiments are needed in the future.

10. CONCLUSIONS

In the first part of this work, the model glycans were successfully analysed using MALDI-MS, leading to a comprehensive qualitative characterization and identification of each glycan.

In the second part, the conditions for separating the model glycans using the HALO® Penta-HILIC column by capLC-UV and capLC-MS were optimised, which resulted in the development of a column test useful for evaluating the performance of the chromatographic system in future analysis of protein glycans derived from glycoproteins of biomedical interest such as hAGP. Nevertheless, the conditions optimised by capLC-UV and capLC-MS did not achieve the separation between MH-ProA and ST-ProA. To go further in this direction, it is necessary to extend this study to assess alternative conditions for ProA-glycans. Despite the current limitations, these preliminary reference methods will still be highly valuable since they provide a standardized approach and can serve as a benchmark for evaluating the performance of the instrumentation used in this study.

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12. ACRONYMS

2-AA: 2-aminobenzoic acid

2-AB: 2-aminobenzamide

AN: Aniline

Asn: Asparagine

CA 19-9: The carbohydrate antigen 19-9

capLC: Capillary Liquid Chromatography

capLC-UV: Capillary Liquid Chromatography coupled with Ultraviolet detection

capLC-MS: Capillary Liquid Chromatography coupled with mass spectrometry detection

CE: Capillary electrophoresis

CHCA: α-Cyano-4-hydroxycinnamic acid

DHB: 2,5-Dihydroxybenzoic acid

DP-15: Maltopentadecaose

DP-14: Maltotetradecaose

DP-13: Maltotridecaose

DP-12: Maltododecaose

DP-11: Maltoundecaose

EIC: Extracted ion chromatogram

ESI-: Negative ion mode electrospray ionization

ESI+: Positive ion mode electrospray ionization

Fuc: Fucose

Gal: Galactose

Glc: Glucose

GlcNAc: N-Acetylglucosamine

GalNAc: N-Acetylgalactosamine

hAGP: Human alpha-1-acid glycoprotein

HILIC: Hydrophilic interaction liquid chromatography

HPLC: High-performance liquid chromatography

LacNAc: N-acetyl-lactosamine

LC: Liquid Chromatography

MALDI: Matrix Assisted Laser Desorption Ionization

Man: Mannose

MH: Maltohexaose

MN: Maltononaose

MS: Mass Spectrometry

m/z: mass charge ratio

NeuAc: N-Acetylneuraminic acid

N/meter: Theoretical plate number relative to the length of the column

PA: 2-aminopyridine

PDAC: Pancreatic ductal adenocarcinoma

Pro: Prolina

ProA: Procainamide

Q-TOF: Quadrupole Time of Flight

RPLC: Reversed-phase liquid chromatography

RSD: Relative standard deviation

Rt : Retention time

Ser: Serine

SiA: Sialic Acid

S/N: Signal-to-noise ratio

ST: LS-Tetrasaccharide a (LSTa)/ Sialyl-lacto-N-tetraose a

THAP: 2',4',6'-Trihydroxyacetophenone monohydrate

Thr: Threonine

TOF: Time Of Flight

UV: Ultraviolet