Off-Line Sample Preparation Techniques for Improved Analysis of Biological Oligosaccharides by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI MS)

by

Sergei I. Snovida

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfilment of the requirements of the degree of

Doctor of Philosophy

Department of Chemistry

University of Manitoba

Winnipeg, Manitoba, Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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Off-Line Sample Preparation Techniques for Improved Analysis of Biological Oligosaccharides by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI MS)

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Of

Doctor of Philosophy

Abstract

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS) is a powerful analytical tool available for studying different biomolecules and their complex mixtures. Special consideration for sample handling, preparation techniques and subsequent analysis by MALDI MS must be taken in order to guarantee the best analysis possible in terms of accuracy and reproducibility of analyte identification, relative and absolute quantification, and the number of mixture constituents detected and characterized. The use of various compounds as matrix additives was found to enhance ion signal intensities of the analyte biomolecules, in particular carbohydrates, over the conventionally used matrix materials. Aniline and *N,N*-dimethylaniline were identified as effective additives to 2,5-dihydroxybenzoic acid, a widely used MALDI matrix, for superior analysis of carbohydrates. Perhaps the most important advantages of using these materials is the fact that sample oligosaccharides need not be derivatized, thus making highly sensitive analysis of native glycans attainable by MALDI MS. Use of these materials also offers some unique advantages, such as a more reliable and reproducible quantitative analysis of sugars and a possibility for designing efficient workflows for automated detection of oligosaccharides in complex biological samples. Multi-dimensional separation procedures, involving various types of liquid chromatography and capillary electrophoresis, were employed for targeted sample fractionation according to modifications of interest, as well as to reduce the signal suppression of the less abundant analytes and to improve the resolution of the sample mixture components. Capillary electrophoresis was demonstrated to be an effective technique for isolating glycopeptides containing sialic acids. Cellulose-based solid phase extraction procedures were found to be extremely useful and efficient for isolation of glycopeptides, and were shown to offer real practical benefits for routine glycoproteomic and glycomic applications. Reversed-phase chromatography was proven to be essential in oligosaccharide purification and in site-specific analysis of protein glycosylation. Examples of the aforementioned techniques and methods with respect to their application to various controlled standard systems, as well as real-life precious biological samples are presented in this work.

Acknowledgements

I would like to thank my supervisor, Dr. Hélène Perreault, for introducing me to the field of biological mass spectrometry and allowing me a complete creative freedom in the nurturing environment of her laboratory. I thank the members of my Ph. D. thesis committee, Dr. Michael S. Freund and Dr. Michael Butler, for their gentle guidance and valuable suggestions in the course of development of my projects. I would also like to thank Dr. John Kelly for serving as my external reviewer. I want to express gratitude to many past and current friends and colleagues at the University of Manitoba, particularly Dr. Vince C. Chen, Dr. Julian A. Saba, Dr. Colin H. Lee, Dr. Oleg Krokhin, Wayne Buchannon, and Dr. Erika Lattova. I thank Dr. Mark E. McComb and Dr. Catherine E. Costello, and other members of the Mass Spectrometry Resource for Biology and Medicine at Boston University School of Medicine, as well as Dr. Kay-Hooi Khoo and his lab members at the Institute of Biological Chemistry of Academia Sinica, for support, advice, and collaborations regarding various aspects of my research and life in general. Many thanks to a large number of individuals, whom I've had a pleasure working and interacting with in the course of my graduate studies, for making my experience most interesting and enjoyable. I would like to acknowledge the Natural Science and Engineering Council (NSERC) of Canada, the Canada Research Chair program (CRC), the Canadian Foundation for Innovation (CFI), and National Science Council (NSC) of Taiwan for funding. Above all, I am grateful to my family and friends, especially my parents Igor and Luba Snovida, for their unconditional love and continuous support: it's priceless…

Thank you!

Sergei I. Snovida

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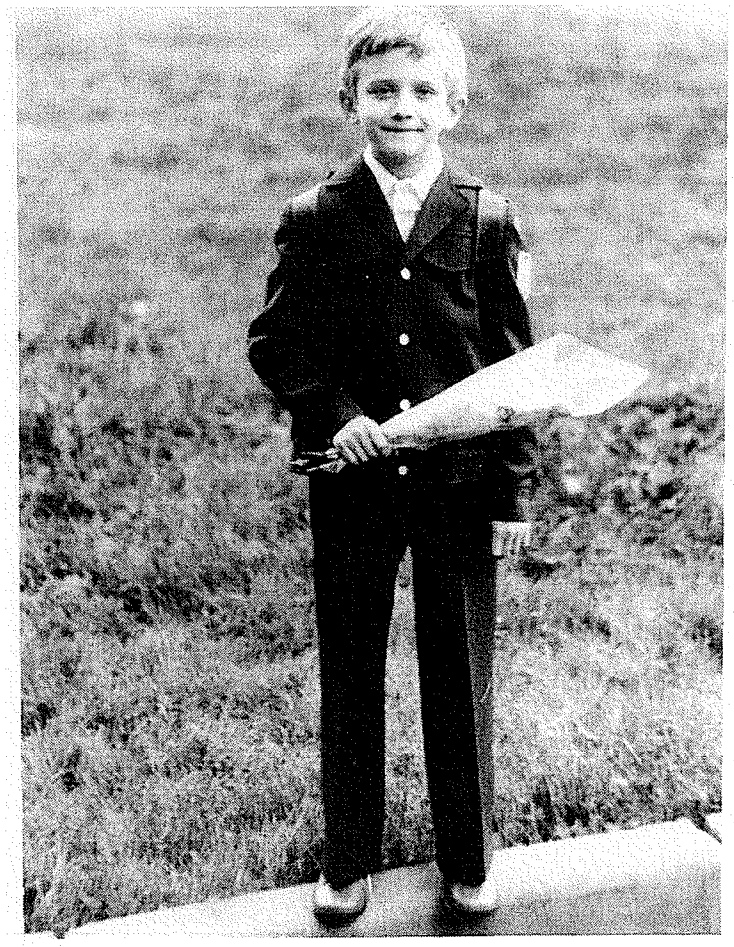
О, сколько нам открытий чудных
Готовят просвещенья дух,
И опыт, сын ошибок трудных,
И гений, парадоксов друг,
И случай, бог изобретатель…

А. С. Пушкин (1829)



This work is dedicated to my loving family and friends.

Thank you!

List of figures

Chapter 1.

Chapter 2.

Chapter 3.

Chapter 4.

Chapter 5.

Chapter 6.

List of tables

Chapter 1.

Chapter 2.

Chapter 3.

Chapter 4.

List of schemes

Chapter 4.

Chapter 6.

Chapter 7.

List of charts

Chapter 4.

Abbreviations

AA	amino acid
ACN	acetonitrile
AN	aniline
α-cyano	α-cyano-4-hydroxycinnamic acid
C_{18}	stationary phase material for reversed-phase chromatography
CE	capillary electrophoresis
CHCA	α-cyano-4-hydroxycinnamic acid
CID	collision-induced dissociation
CZE	capillary zone electrophoresis
Da	Dalton
DE	delayed extraction
DHB	2,5-dihydroxybenzoic acid
DMA	*N,N*-dimethylaniline
DTT	dithiothreitol
EOF	electroosmotic flow
ESI	electrospray ionization
ETD	electron transfer dissociation
Fuc	fucose
Gal	galactose
Glc	glucose
GlcNAc	N-acetylglucosamine
H_2O	water
HPLC	high performance liquid chromatography

IAA	iodoacetamide
IT	ion trap
L	litre
M	
LIF	laser-induced fluorescence
M	molar, mol/L
M_n	number average molecular weight
M_p	peak average molecular weight
MALDI	matrix-assisted laser desorption ionization
Man	mannose
MeOH	methanol
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MW	molecular weight
m/z	mass-to-charge ratio
Neu	5-*N*-acetylneuraminic acid
Neu5Ac	5-*N*-acetylneuraminic acid
Neu5Gc	5-*N*-glycolylneuraminic acid
NMR	nuclear magnetic resonance
NP	normal phase
PAGE	polyacrylamide gel electrophoresis
PH	phenylhydrazine
pI	isoelectric point
pMe	permethylated
PSD	post-source decay

PTM	post-translational modification
Q	quadrupole
RP	reversed-phase
SA	sialic acid
SDS	sodium dodecyl sulfate
SPE	solid phase extraction
S/N	signal-to-noise ratio
TFA	trifluoroacetic acid
TOF	time-of-flight
UV	ultraviolet

List of publications

(1) **Snovida, S. I.**, Rak-Banville, J. M., Perreault, H., On the Use of DHB/Aniline and DHB/*N,N*-dimethylaniline Matrices for Improved Detection of Carbohydrates: Automated Identification of Oligosaccharides and Quantitative Analysis of Sialylated Glycans by MALDI-TOF Mass Spectrometry, *J. Am. Soc. Mass Spectrom.* **2008**, *19* (8), 1138-1146

(2) **Snovida, S. I.**, Perreault, H., A 2,5-Dihydroxybenzoic Acid/*N,N*-Dimethylaniline Matrix for the Analysis of Oligosaccharides by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry, *Rapid Commun. Mass Spectrom.* **2007** *21* (22), 3711-3715

(3) **Snovida, S. I.**, Chen, V. C., Perreault, H., Use of a Novel DHB/Aniline MALDI Matrix for Improved Detection and On-Target Derivatization of Glycans: a Preliminary Report, *Anal. Chem.* **2006** *78* (24), 8561-8568

(4) **Snovida, S. I.**, Chen, V. C., Krokhin, O., Perreault, H., Isolation and Identification of Sialylated Glycopeptides from Bovine alpha(1)-Acid Glycoprotein by Off-Line Capillary Electrophoresis MALDI-TOF Mass Spectrometry, *Anal. Chem.* **2006** *78* (18), 6556-6563

(5) Lattova, E., **Snovida, S. I.**, Perreault, H., Krokhin, O., Influence of the labeling group on ionization and fragmentation of carbohydrates in mass spectrometry, *J. Am. Soc. Mass Spectrom.* **2005** *16* (5), 683-696

Chapter 1:

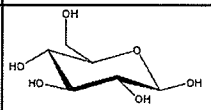
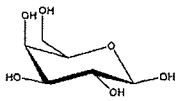
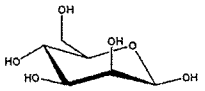
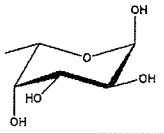
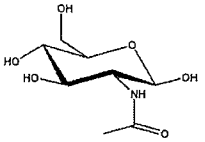
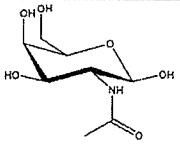
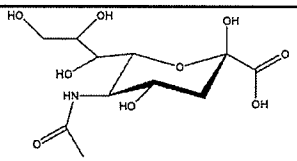
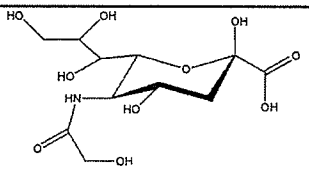
General Introduction.

1.1 Carbohydrates in biology

Monosaccharides are the most fundamental units of carbohydrates – an important class of molecules most often referred to simply as sugars. The terms "glycan" and "oligosaccharide" are frequently used interchangeably to refer to polymers of monosaccharides, which represent more complex carbohydrate structures. In some instances, the term "glycan" is also used to describe a carbohydrate portion of a multipart biomolecule. Some of the most common monosaccharides that make up glycans in biological systems are listed in Table 1.1.

Glycobiology is a field in biology devoted to the study of the structure, biosynthesis, and biological function of sugars occurring in nature. In addition to free saccharides, there is a large number of glycoconjugates found in a wide range of organisms, in which sugars are associated with proteins, lipids, and nucleic acids. Glycomics refers to the process of careful examination of all glycan structures of a given cell type, tissue, or an organism. The term "glycoproteomics" is used in the context of studies related to glycomes of glycoproteins, specifically in terms of structure/function relationship between the joint glycan and polypeptide components, as well as their functional interactions with other biomolecules[1].

Table 1.1. Common monosaccharides encountered in glycoproteins.

Monosaccharide	Structure	Abbreviation	Symbol[a]	Anomers	Linkages
D-glucose		**Glc**		α	2,3
D-galactose		**Gal**		α β	3 3,4,6
D-mannose		**Man**		α β	2,3,6 4
L-fucose		**Fuc**		α	2,3,4,6
N-acetyl-D-glucosamine		**GlcNAc**		β	2,3,4,6
N-acetyl-D-galactoseamine		**GalNAc**		α β	3 4
5(*N*)-acetyl neuraminic acid (a sialic acid)		**Neu5Ac**		α	3,6,8
5(*N*)-glycolyl neuraminic acid (a sialic acid)		**Neu5Gc**		α	3,6,8

[a]Note that at present, there is no universal official convention for the use of symbols to represent monosaccharide structures. These are usually defined in the context of a presentation. The symbols used here are the most encountered in most publications.

Glycoproteomic studies currently represent an area of active research in life sciences[1-5]. A particular challenge innate to this field stems from the enormous complexity of oligosaccharide structures as a consequence of the elaborate processes

regulating their biosynthesis. Unlike proteins and nucleic acids, these molecules are non-linear and may branch at several locations on each monosaccharide unit. Modifications such as sulfation, phosphorylation, acetylation, among others, also make detailed and accurate structural analysis of glycans extremely difficult. In addition to this, a very broad variability of glycan structures within a given glycoprotein is common. For this reason, protein-specific glycomes are often described in terms of occurrence and distributions of different glycan structures, or glycoforms, which may change depending on the developmental and/or pathological state of an organism or in response to environmental stresses.

The biological importance of glycoproteins and the need for continuous development of new analytical tools in the realm of glycoproteomics and glycomics have been emphasized heavily throughout current literature. Glycosylation of proteins is an important type of post-translational modification. The biological activity of many important biomolecules depends on the extent and nature of their glycosylation in terms of site occupancy and glycan structure variability. Changes in glycosylation may also be associated with a pathological or oncological state of an organism, and thus elevated or diminished levels of certain glycoforms may be used as biomarkers for disease[6].

In general, there are two types of protein glycosylation encountered in nature, the *N*-linked and the *O*-linked types[1,2]. *N*-linked glycans are attached to proteins via the amide nitrogen of asparagine, whereas *O*-linked glycans are attached through the side-group oxygen of serine and threonine. The attachment site on a glycan moiety is termed the "reducing end" of a glycan. *O*-linked glycans are usually more challenging to deal with because of broad diversity in their structures and lack of adequate enzymatic or

chemical means of their release from the protein. Analysis of *N*-linked glycans, on the other hand, is made easier by the readily available enzymatic methods for their release from proteins, common core structures (Figure 1.1), and their occurrence at a specific consensus sequence, a sequon, along the polypeptide chain (asparagine-X-serine/threonine, where X is any amino acid except proline), which may be predicted from genomic or proteomic sequences. The vast majority of research in glycoproteomics has thus far been focused on analyzing of *N*-linked glycans[2,7].

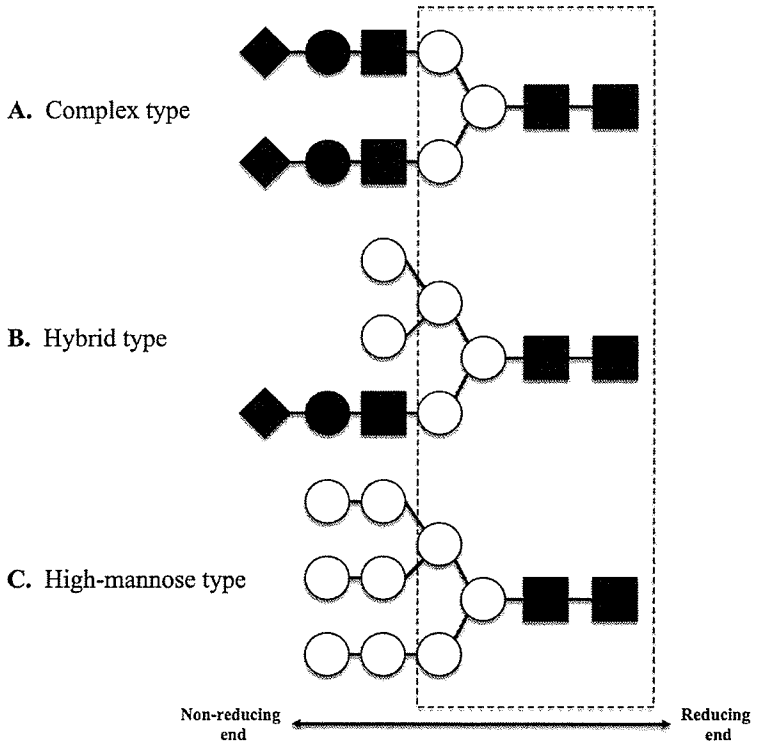


Figure 1.1. The general features of *N*-linked glycans.

1.2 Tools for the analysis of glycans

Several approaches and techniques have been developed and successfully utilized for analysis of glycans and glycoconjugates over the years. Chromatographic and electrophoretic separation methods have been used to resolve complex mixtures of these biomolecules for the purpose of detection and quantitation of the individual components[5,8,9]. Studies using nuclear magnetic resonance spectroscopy (NMR) and enzyme-based molecular biology methods also have been and still are frequently used for structural elucidation[10,11]. Most of these techniques, however, rely on fairly large sample quantities to make sensitive analysis possible, or demand that substantial derivatization be performed on samples for compatibility with a particular technique. Given that many biological samples are very scarce, expensive, and are very difficult to obtain on large scale, the analysis of samples by such techniques may not always be feasible.

1.2.1 Mass spectrometry

At present, mass spectrometry (MS) is a central tool at the forefront of glycoproteomic and glycomic research, as it provides practically unparalleled precision and sensitivity related to protein glycosylation analysis and structural studies of glycans. MS-based workflows allow for accurate identification of glycoproteins found within complex biological matrices, determination of glycosylation sites, as well as detailed analysis of the individual glycoforms in terms of composition, relative abundance, and exact structure[1-5].

Since the initial introduction and subsequent commercialization of electrospray ionization (ESI)[12] and matrix-assisted laser desorption/ionization (MALDI)[13,14] instruments in the 1980's, which made stable ionization of large, often labile biomolecules in the gas phase possible, mass spectrometric analysis of glycans and their glycoconjugates has become a standard for detection and characterization purposes. Very small sample amounts, ranging from low nanomole to mid-femtomole quantities, are routinely analyzed using MS instruments.

The essence of mass spectrometry is separation and mass measurement of small amounts of ions in gas phase. This is accomplished by first ionizing analyte molecules and followed by the measurement of their masses. Since ions are charged, the actual parameter being measured is mass-over-charge ratio, *m/z*, where *m* is the molecular weight of the ion and *z* is its charge state. Positive and negative ions may be created through the addition of protons/cations, or elimination of protons and addition of anions, respectively. Both types may be detected by alternating the electric voltage polarity of the instrument. Thus, instruments generally consist of these components: an ion source, where sample is ionized, a mass analyzer, the module performing the actual mass measurement, and a detector. Analyte molecules are identified on the basis of accurate mass measurements provided by a mass spectrometer. By far the most common ion sources for biomolecules are ESI and MALDI. Each type has its own advantages as well as limitations.

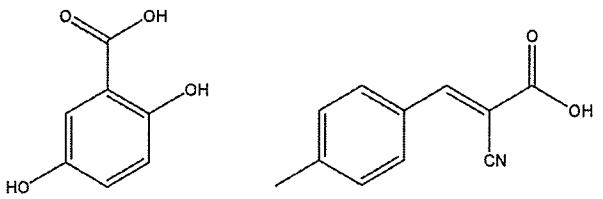
1.2.1.1 ESI mass spectrometry

Electrospray sources ionize molecules from solution state and are continuous, i.e.

ions are generated constantly during spraying. For this reason, they are normally coupled to quadrupole or ion trap mass analyzer[12]. A distinguishing feature of this technique is production of multiple-charge states during ionization and the mass spectra are characterized by occurrence of several peaks at different *m/z*, each corresponding to a different *z* integer value. The most important advantages of ESI-based instruments are (i) they allow detection of very large molecules as multiply charged ions, and (ii) their compatibility with on-line coupling to liquid-phase separation techniques, such as liquid chromatography (LC) or capillary electrophoresis (CE).

1.2.1.2 MALDI-TOF mass spectrometry

In MALDI, the sample is ionized via electrochemical reactions between the analyte and matrix material, which is excited by a laser. The most common substances used as MALDI matrices are 2,5-dihydroxybenzoic acid (DHB) and α-cyano-4-hydroxycinnamic acid (CHCA). Their structures are given in Figure 1.2.



DHB **CHCA**

Figure 1.2. Some common MALDI matrices.

The MALDI process is depicted in Figure 1.3. A sample is deposited onto a target plate made of a conducting material (typically stainless steel) in a concentrated solution of matrix substance. When the mixture dries, the resulting sample/matrix crystals are irradiated with a laser at a wavelength of high absorbance by the matrix compound, which is typically in the 300 nm ultraviolet (UV) region. MALDI events take place on a very short time-scale. Each laser pulse leads to absorption of energy by the matrix, which is ablated from the surface in a form of a very dense, high-temperature plume of gas consisting of matrix and analyte in molecular and ionic forms. An intricate cascade of gas-phase reactions ultimately leads to ionization of the analyte.

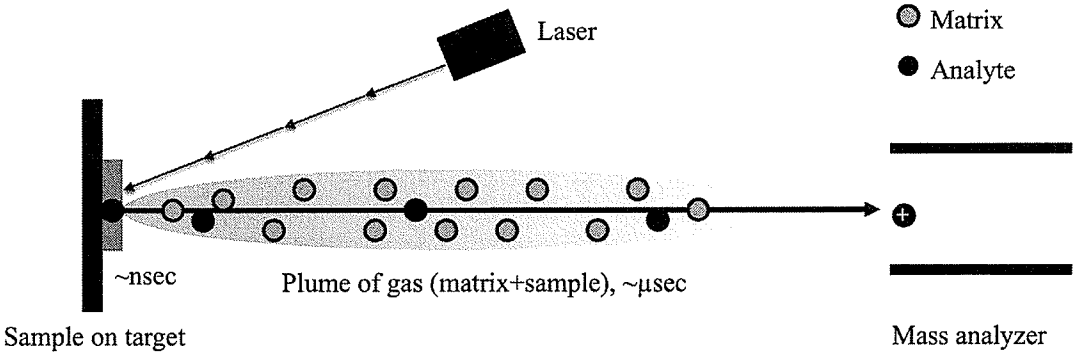


Figure 1.3. The MALDI process[13,14].

Due to its pulsed character, MALDI is naturally coupled to time-of-flight (TOF) mass analyzers. Mass analysis by TOF is illustrated in Figure 1.4. Upon ionization, ions enter a TOF tube with equal kinetic energy (KE), which is determined by the ion's mass (*m*), velocity (v), and charge (*z*). Ions of different *m/z* values will travel through the tube at different velocities, i.e., v is inversely related to *m/z*. Therefore, ions with high *m/z* will pass through the TOF tube of length *L* and arrive at the detector later than the ions of lower *m/z*. Ultimately, the ions time-of-flight is used to calculate its *m/z* value.

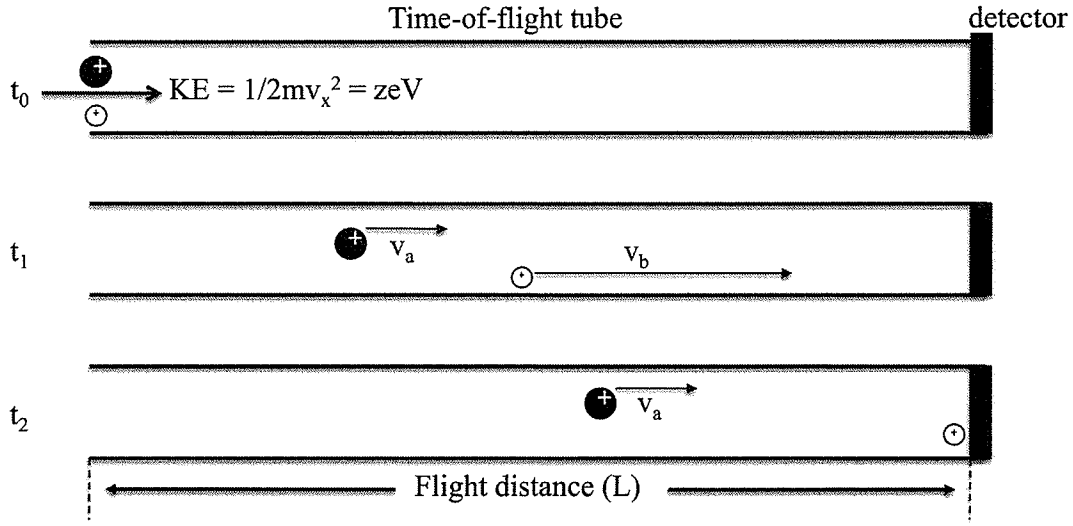


Figure 1.4. Time-of-flight mass measurement. The velocity of ions having a higher *m/z* value is denoted by V_a; V_b denotes the velocity of ions with a lower *m/z* value.

The main advantage of MALDI is the production of mostly singly-charged ions, which makes spectral assignment very easy. However, the on-line coupling of separation techniques to this ionization mode is not particularly straightforward. Despite this limitation, MALDI is often the top choice MS technique for initial sample profiling and for detailed structural analysis in glycoproteomic/glycomic workflows[7].

1.2.1.3 MS/MS fragmentation

A simple mass spectrometry experiment only provides accurate masses for sample components, which may be used to identify each compound. However, by itself this is not sufficient, as there are many structural isomers or isobaric compounds that occur in nature and these are not differentiated by mass. Additional information pertaining to connectivity of individual atoms or functional groups in a molecule is required in order to unequivocally establish the identity of a given ion. Tandem MS, or MS/MS experiments are often done to confirm the identity and to provide detailed structural information related to a given analyte[2,15,16]. Several modes of fragmentation exist today, including collision-induced dissociation (CID), electron-capture dissociation (ETD), infrared multiphoton dissociation (IRMPD), as well as several others, however collision-induced dissociation (CID) is the most commonly available method. In CID, ions of a selected *m/z* value are introduced into a collision cell pressurized with inert gas (typically N_2 or He). There, ions undergo collisional activation (absorption of collisional energy), which is dissipated through fragmentation of the parent ion into several smaller stable fragments. A back-end mass analyzer then measures these fragments. Because fragment ions are often structure-specific, a unique fingerprint may be generated for a given analyte species. Moreover, these fragments may be used to "stitch together" the original structure of the molecule.

A general fragmentation pattern for carbohydrates is presented in Figure 1.5 according to the nomenclature proposed by Domon and Costello[17]. The excitation energy imparted onto a molecule by collisional activation is dissipated through excessive electronic and vibrational activity, which eventually leads to rupture of the covalent

bonds made weak by instantaneously favourable electronic arrangement at certain locations within a molecule.

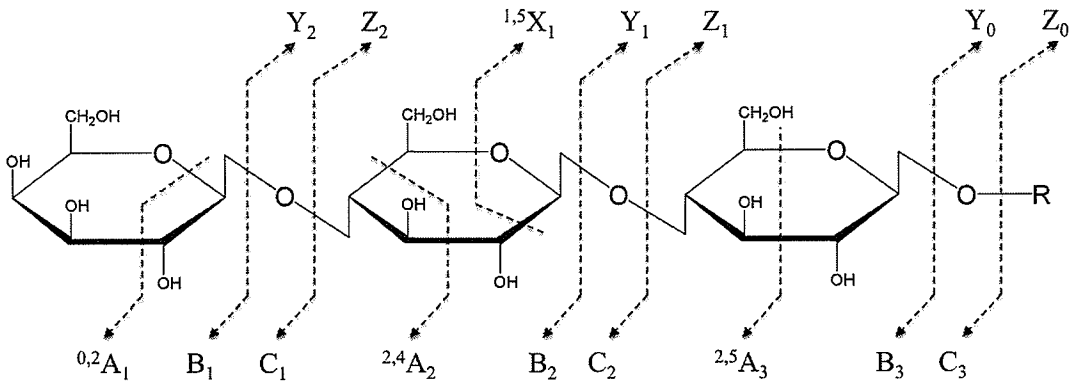


Figure 1.5. Fragmentation patterns of carbohydrates[17].

Following the electronic rearrangements and/or further fragmentation, a stable daughter fragment is produced. Depending on the energies involved in activation, different fragment types are possible. Low (eV) and high (keV) energy CID fragmentation experiments are achievable, although the high energy CID is available exclusively on certain sector mass spectrometers and TOF/TOF-type instruments. Fragmentation of glycopeptides is depicted schematically in Figure 1.6. Under low energy CID conditions, the peptide backbone typically fragments at the weaker peptide bond into b/y-ion series. The glycan part is usually cleaved off *en bloc* to produce depicted peptide ions.

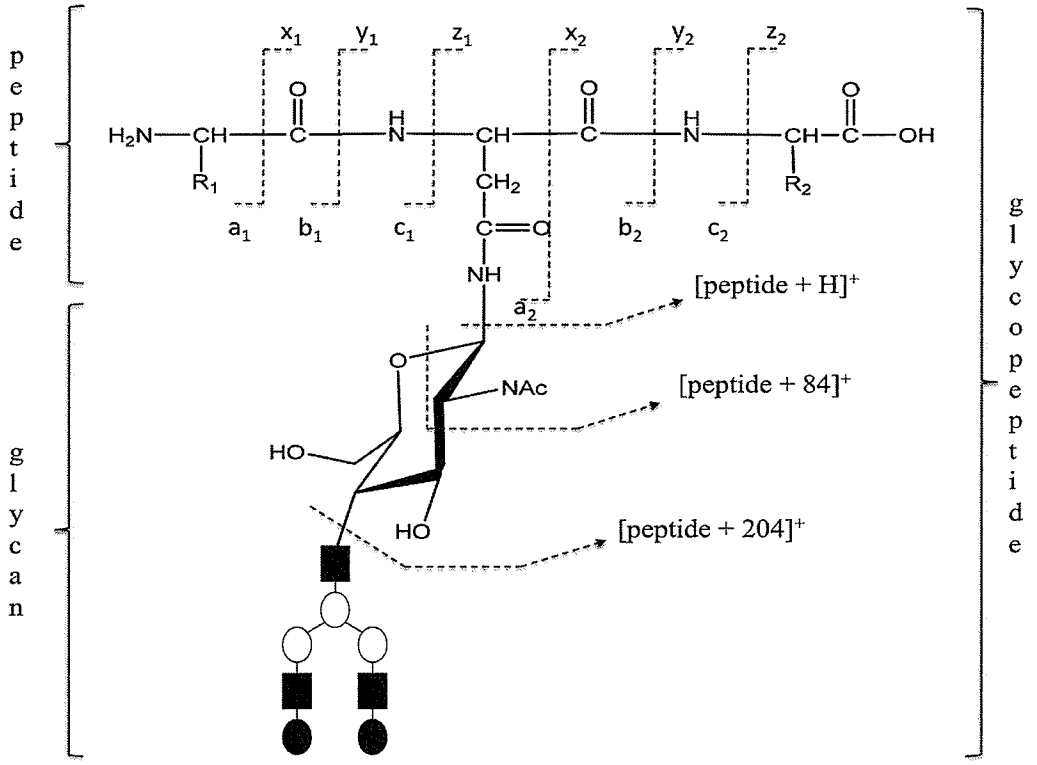


Figure 1.6. Fragmentation of glycopeptides.

A typical MALDI-qTOF CID spectrum of a singly charged *N*-linked glycopeptide is given in Figure 1.7. Three distinct regions are observed here. The main signature of an *N*-glycopeptide is the occurrence of a high intensity triplet of peaks corresponding to $[peptide + H]^+$, $[peptide + {}^{0,2}X\ GlcNAc\ fragment]^+$, and $[peptide + GlcNAc]^+$ ions. These ions, together with ions resulting from the loss of monosaccharides at the non-reducing end, allow identifying the glycan in terms of its monosaccharide composition. The lower *m/z* region contains peptide fragmentation ion series, hence the peptide may be sequenced, and the position of glycosylation determined by carefully examining this region of the spectrum[15].

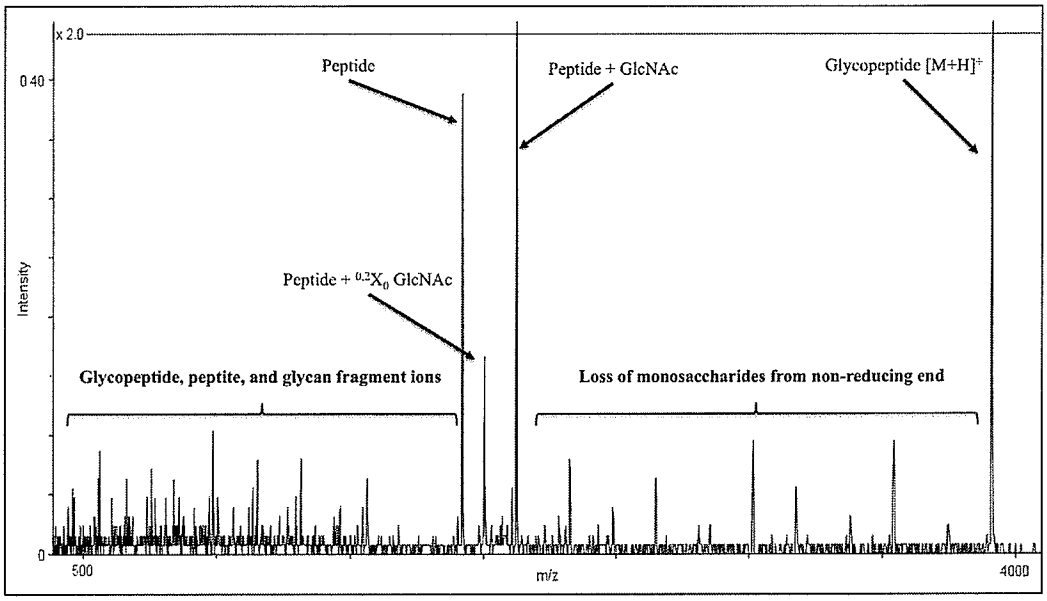


Figure 1.7. A typical MALDI qTOF CID spectrum of a singly charged *N*-linked glycopeptide.

1.2.2 Separation techniques

Satisfactory analysis may often be achieved by using one of several separation techniques coupled to a sensitive non-MS detector. The two main types of separation used are based on liquid chromatography and capillary electrophoresis, which operate on very different principles. Spectroscopic detection based on absorbance and/or fluorescence is used extensively in glycomic and glycoproteomic workflows. For the analysis of glycans, labeling with a good chromophore or a fluorophore is a must, since carbohydrates do not absorb appreciably well in the UV to facilitate sensitive detection[18,19]. A general scheme for reductive amination, a common labeling procedure of glycans, is depicted in Figure 1.8. Separation techniques coupled to spectroscopic detectors are ideal for the quantitative analysis of glycans, since the detected signal is

entirely due to the common label with a constant molar absorptivity coefficient, which is completely independent of the glycan moiety attached. However, the main limiting factor of UV/fluorescence-based analyses is that standards must be available for each analyte in order to identify the unknowns on the basis of retention time. This may not always be practically feasible.

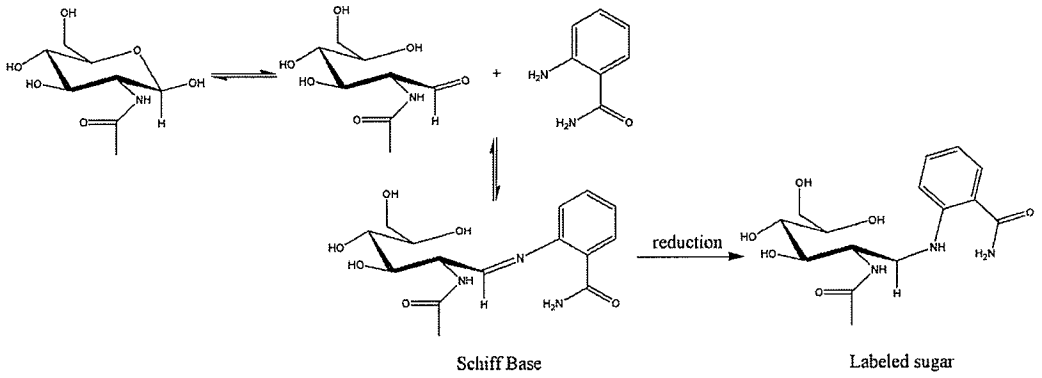


Figure 1.8. A general scheme for reductive amination of glycans.

Peptides on the other hand, do exhibit appreciable spectroscopic activity in the UV due to the presence of chromophores in amino acids such as tryptophan, as well as the presence of amide groups, and for this reason glycopeptides generally need not be derivatized prior to analysis[15]. However, quantitative analysis may not be performed here due to differences in molar absorptivities of individual components at a given wavelength. Moreover, it is virtually impossible (from a practical standpoint) to have standards for each component found in the proteolytic digest of a complex biological sample mixture.

For the reasons mentioned earlier, separation techniques are most frequently employed in conjunction with on-line MS detection or sample purification/fractionation prior to MS analysis in the context of glycoproteomic/glycomic workflows. Off-line separation coupling to MALDI MS is also widely used[20,21]. These procedures usually lead to a dramatic increase in sensitivity towards low-abundance compounds of interest and greatly simplify overall interpretation of the mass spectra.

1.2.2.1 Capillary electrophoresis

Electrophoretic separations are based on differential rates of migration of analytes of different charge and size. Capillary electrophoresis is one variation of the techniques of this type[22,23]. A general CE setup is depicted in Figure 1.9. The sample is introduced at one end of a small diameter fused-silica capillary (~ 10-100 μm) filled with a running buffer electrolyte solution. Both capillary ends are then submerged into vials filled with the running electrolyte and a potential is applied across the capillary. Charged sample components migrate towards electrodes of opposite polarity. The individual rate of migration depends on charge, molecular cross-section, and size, which essentially describe the charge density of an analyte. Ions having identical above characteristics form narrow zones within the capillary in the course of a CE run. Additionally, electroosmotic flow (EOF), a phenomenon created by polarization of silanol groups inside the capillary and resulting ionic currents, moves the bulk liquid towards the anodic end. Thus, the net migration of analytes is a sum of the individual migration of analyte in the potential field and electroosmotic flow. Because of EOF, neutral, basic, and acidic species may be separated and detected in a single run by carefully manipulating factors that control

electroosmotic flow and the individual mobilities of analytes. Very high resolving power and separation efficiency may be attained by the use of capillary electrophoresis techniques. However, the main drawbacks associated with CE are related to its extreme sensitivity to sample impurities, which often render a given method unusable, and to delivery of only very small samples quantities, which may be inconvenient for some applications in biology.

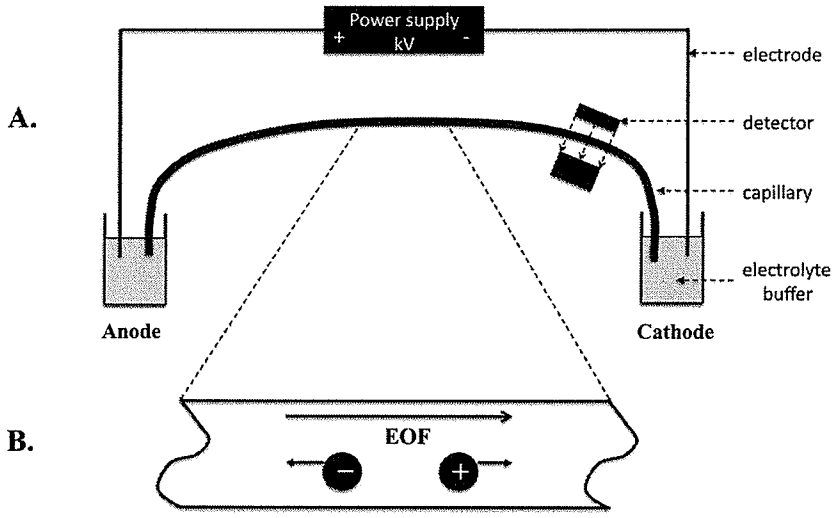


Figure 1.9. General setup of a CE apparatus. In this figure, the sample injection is done at the anodic end.

1.2.2.2 Liquid chromatography

The most effective and widely used separation techniques in MS based glycoproteomic and glycomic studies involve liquid chromatography (LC). The application range of these techniques is very broad and covers anything from sample purification and fractionation, to more recent retention-based peptide sequence

prediction[21]. The advantages include relative simplicity of method development, ruggedness relative to samples types handled, an ability to accommodate large sample volumes/quantities, reproducibility, and the range of available stationary phase materials.

Separations are based on differential partitioning of analytes between solid stationary phase and liquid mobile phase. Partitioning depends on the strength of interactions, which may be ionic, hydrophobic, hydrophilic, or of any other affinity mode. Chromatography generally refers to separation of all individual sample components in a continuous manner, whereas solid-phase extraction (SPE) procedures aim at targeted "one-step" extractions of compounds of interest from a sample mixture. Both chromatography and solid-phase extraction methods are ubiquitous in preparative and analytical workflows in biological sciences. Coupled to sensitive MS instruments, these workflows persistently advance the level of our understanding of structure and function of the biomolecules that govern life.

1.3 Overall research theme

The work presented in this thesis consists of two very independent projects unified by a common research objective, which was to improve sensitivity in mass spectrometric detection and characterization of biological oligosaccharides. The efforts demonstrate that this may be accomplished at two levels: (i) sample preparation and purification (project 1), and (ii) ionization of sample (project 2). Thus, availability of higher amounts of purer sample material, as well as implementation of ionization techniques that result in production of more analyte ions, will lead to more sensitive analyses overall.

The first project, as outlined in Chapers 2 and 3, dealt with targeted isolation of glycopeptides by capillary electrophoresis and cellulose-based solid-phase extraction procedures. The overall objective there was to develop methods for extraction of either all glycopeptides (Chapter 3) or only those containing acidic residues in their glycoforms (Chapter 2) in order to provide a more sensitive glycoproteomic/glycomic MS analysis at the glycopeptide level. Examination of samples that contain only analytes of interest greatly simplifies mass spectral interpretation. Additionally, the ionization efficiency of low-abundance analytes improves as a consequence of reducing the amount of suppressing species in the sample matrices.

The second project (Chapters 4-6) explored the effects of modifying MALDI matrix materials to improve glycan sensitivity at the ionization level. If more ions are created by a MALDI event, more ions will in turn enter the mass analyzer, which would result in a stronger signal. Ionization of native glycans in the gas phase is particularly difficult due to their hydrophilic nature. Moreover, the application of higher ionizing energies to overcome this problem usually leads to undesirable fragmentation of parent ions, thus diminishing signal quality and introducing false positives into the analysis. By using matrix modifiers in conventional MALDI matrix preparations and developing new sample/matrix crystallization methods, it is possible to significantly enhance the signal quality of native oligosaccharides in MALDI MS, as demonstrated in Chapters 4-6.

Each chapter contains a detailed introduction section tailored to the specific topics explored, instruments used, and experiments performed. All work presented in this thesis has either been already published[24-27] or is being written up in preparation for a journal submission. In the concluding Chapter 7, the work is discussed retrospectively in terms of

various advantages and limitations. That section also offers a perspective on the current state of the field and provides suggestions related to directions of future research goals.

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Chapter 2:

Development of CE-based technique for isolation of sialylated glycopeptides and subsequent analysis by MALDI MS.

2.1 Authors contributions

All experiments pertaining to this work were designed and performed by Sergei I. Snovida under the guidance of Dr. H. Perreault. Dr. O. Krokhin was responsible for acquisition of data on the MALDI-QqTOF instrument. Sergei I. Snovida was also responsible for manuscript drafts and all figures. Dr. V. C. Chen provided help with liquid chromatography separations. Dr. H. Perreault was responsible for editing the final version of the manuscript prior to initial submission to the journal of Analytical Chemistry and providing the funding for this research. All authors contributed equally to final revision of the manuscript prior to its publication.

Isolation and Identification of Sialylated Glycopeptides from Bovine α_1-acid Glycoprotein by Off-line Capillary Electrophoresis MALDI-TOF Mass Spectrometry

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Published: *Anal. Chem.* **2006**, *78* (18), 6556-6563

2.2 Abstract

Sialylated glycopeptides contained in liquid chromatographic fractions of bovine α_1-glycoprotein tryptic digests were isolated from asialo peptides using capillary electrophoresis (CE). CE effluents were deposited directly onto a metallic target and analyzed using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry. This method allowed the characterization of four *N*-glycosylation sites in the glycoprotein and each site was observed as a set of sialylated peptide glycoforms. Tandem mass spectrometry was used to confirm peptide sequences and glycan content in glycoforms. The CE method was developed for this study resulted in a very clear separation of the sialylated from the asialo content of glycoprotein digests and proved very useful in the determination of the nature and location of sialylated glycans along the protein chain. This article is the first report describing the use of on-target CE fraction collection using a MALDI removable sample concentrator.

2.3 Introduction

Sialic acids are important components of glycans found in different types of glycoconjugates in a variety of organisms. They are acidic sugars involved in many key biological phenomena, including cell-cell adhesion and cell-pathogen interaction, and are typically found at the outmost regions of glycans[1-3]. Their expression is often dependent on the cell type, developmental stage of an organism, or a disease state. More than 40 types of sialic acids have been identified over the years. The differences arise from modifications such as acetylation, glycolylation, and methylation at different locations in the sugar. One common characteristic shared between all sialic acids is the presence of a carboxyl group at the anomeric carbon, which is usually ionized at physiological pH, thus resulting in a negative charge above pH value of about 2.6.

Many glycoproteins have pI values significantly lower than their deglycosylated forms. This is due to negatively charged sialic acid residues found in the glycan structures on these proteins. Alpha-1-acid glycoprotein, an acute phase protein, was among the first such acidic glycoproteins identified in mammalian blood sera[4,5]. It has been studied extensively over the years in several organisms and its glycosylation sites and patterns are reasonably well known today[6-8]. This protein contains five *N*-glycosylation sites with complex-type glycan structures and as a result of a large amount of knowledge available pertaining to this protein, it is often used as a model compound in analyses of biologically-derived oligosaccharides and glycopeptides for the purpose of developing or evaluating new analytical procedures[9-11]. Almost all methods which are used to characterize glycoproteins and their oligosaccharides involve use of separation techniques based on electrophoresis and liquid chromatography (LC) followed by further

analysis by mass spectrometry (MS)[12,13]. Researchers are typically limited to only small amounts of biological sample, and MS is an ideal tool for structural analysis of scarce biological compounds.

Capillary electrophoresis (CE) is an excellent separation technique for glycomic and proteomic analysis[14]. It requires minimal amounts of materials, including sample and electrolyte solutions. Several studies report efficient separation of various glycoforms found in glycopeptide and oligosaccharide mixtures using this technique[15,16]. CE may be used as a second dimension to LC separations, leading to improved overall resolution of sample components, and is extremely useful in site-specific glycopeptide analysis. While many such methods demonstrate enhanced resolution of various glycoforms, the use of different detergents and salts often renders these procedures incompatible with subsequent analysis by MS. In this manuscript we report on an efficient CE-based technique for isolating sialoglycopeptides from tryptic digest mixtures of bovine α_1-acid glycoprotein for off-line analysis by matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS. This article is the first report to describe the use of on-target CE fraction collection using a MALDI removable sample concentrator and making use of the metallic target as an electrode.

2.4 Experimental

2.4.1 Materials

Bovine α_1-acid glycoprotein and 2,5-dihydroxybenzoic acid (DHB) were purchased from Sigma (St. Louis, MO, USA), sequencing-grade trypsin and PNGase F enzymes were obtained from Promega (Madison, WI, USA) and ProZyme (San Leandro, CA, USA), respectively. Milli-Q water was used in preparation of all solutions. C_{18} chromatographic packing material used for reverse-phase chromatography separations (WP C_{18} Prep. HPLC Bulk Packing 15 μm, 300 Å) was purchased from J.T. Baker (Phillipsburg, NJ, USA), peptide standards ($ACTH_{1-17}$, Angiotensin II, Bombesin, Substance P) were obtained from American Peptide Company (Sunnyvale, CA, USA).

2.4.2 Sample preparation

Digestion of α_1-acid glycoprotein with trypsin was performed by dissolving 0.5 mg of the protein in 1 mL of trypsin solution (10 ng/μL in a 25 mM ammonium bicarbonate buffer, pH 7.8) in a 1.5 mL Eppendorf™ tube. The final enzyme to substrate ratio was 1:50. The solution was incubated for 18 hours at 37°C. Digestion was stopped by adding 100 μL of 0.1% trifluoroacetic acid (TFA) solution. The solution was lyophilized and material was re-suspended in 100 μL of water.

To remove sialic acids from the sample glycopeptides, 90 μL of 0.05 M H_2SO_4 were added to 10 μL the tryptic digest in a 0.5 mL Eppendorf™ tube and the mixture was incubated at 80°C for 60 minutes with frequent shaking. Upon completing of the hydrolysis reaction, the solution was reduced to 20 μL using a Speed Vac system. This preparation will be referred to as "asialo sample".

N-linked glycans were removed from α_1-acid glycoprotein by treating a 10 μL portion of the final tryptic digest solution with the recommended amount of PNGase F (as per manufacturer's manual) in a phosphate buffer provided in a 0.5 mL Eppendorf™ tube. The reaction was allowed to go for 18 hours at 37°C. Upon completion of the reaction, the contents were lyophilized and re-suspended in 20 μL of water. This sample is referred to as the "deglycosylated sample" in this manuscript.

2.4.3 Liquid chromatography separations

The three sets of tryptic digests solutions (untreated, asialo, and deglycosylated) were each separated into 30 fractions by reversed phase chromatography using a SepDep device[17]. LC columns were prepared by packing C_{18} chromatographic material into the midsection of a GELoader (Eppendorf) tip (~50 μL). A small piece of a Kimwipe® was inserted into a GELoader tip and pushed firmly into the narrow end to prevent the packing material from "leaking out" from the column. Packing material suspended in methanol was then added slowly to the GELoader tip under vacuum suction in small increments, allowing the material to settle after each addition. Upon completion of this step, the column was conditioned by first washing it with a 0.1% TFA in a 1:1 water-acetonitrile solution (100 μL), followed by a wash with a solution of 0.1% TFA in water (100 μL). After loading of the sample (20 μL), the column was washed with two 50 μL portions of 0.1% TFA in water solution in order to remove any digestion buffer salts and H_2SO_4 present in the sample. Fractions were collected with an acetonitrile-water step-gradient (50 μL/fraction; 2.5% increase in acetonitrile at each 2 fractions; 2.5-50% acetonitrile in 0.1% TFA water).

2.4.4 Capillary electrophoresis separations

CE experiments were performed on a Beckman P/ACE 5000 CE instrument equipped with a UV detector. Untreated fused silica capillaries (360 μm OD; 50 μm ID; 80 and 120 cm total length, 60 and 100 cm to the detector, respectively) were used. The ammonium acetate running electrolyte was prepared by adjusting the pH of a 0.5% aqueous acetic acid solution to 4.7 with concentrated ammonium hydroxide solution. Material from each LC fraction was lyophilized and dissolved in 10 μL of 1% acetic acid solution (pH 2.8). About 100 nL of sample solution were pressure-injected into the capillary. This was followed by additional injection of 100 nL of running electrolyte at the inlet end. Separations were performed in the forward polarity mode at 10-15 kV and the capillary was kept at 20°C. Electropherograms were acquired at 214 nm.

A 96-well BD^{TM} MALDI sample concentrator device[18] (BD Biosciences, Bedford, MA, USA) was evaluated for use in collecting fractions directly onto a MALDI target in the CE separation experiments. The device is an elastomeric 96-well plate with conical, tapered wells open at both ends. The device is placed over a MALDI target, which is grounded, the side with the narrow end of the well facing the target. As poly(dimethylsiloxane) (PDMS) adheres well to the stainless steel MALDI target, the wells are filled with a CE running buffer and the outlet end of the capillary is placed into a well. As capillary electrophoresis is being performed, the capillary may be manually moved from one well to next, thus collecting fractions on the target, used as an electrode. The material in each well is then evaporated and MALDI matrix solution is added into each well. Upon evaporation of the solvent, the matrix with co-crystallized sample material is drawn down onto the MALDI target. The device is then removed from the

target, leaving 2 mm diameter sample spots. The fractions are then subjected to analysis by MALDI-TOF-MS. This method of CE fraction collection was utilized in the experiments described in this manuscript.

2.4.5 Mass spectrometric measurements

All single mass spectra were acquired on a Bruker Biflex IV MALDI-TOF mass spectrometer in the positive ion reflecting mode. MS/MS experiments were performed on the University of Manitoba/Sciex MALDI-QqTOF instrument (Department of Physics, University of Manitoba, Canada; PE Sciex, Mississauga, Ontario, Canada).

2.4.6 Online tools

The amino acid sequence of bovine α_1-acid glycoprotein was obtained from the NCBI protein database[19] (accession # CAH59718). NetNGlyc 1.0 tool[20] was used to analyze the sequence of bovine α_1-acid glycoprotein for potential *N*-glycosylation sites. *In silico* digestion of the protein and calculation of the pI values for the peptides were performed using PeptideMass and Compute pI/MW Tool, respectively, from ExPASy proteomic tools[21]. CE Expert Lite online tool[22] was used in calculation of sample injection volumes for CE experiments.

2.5 Results and discussion

2.5.1 Preliminary work

In our study, we initially set out to investigate the effects of glycosylation on migration of peptides under CE conditions that are compatible with subsequent off-line

analysis by MALDI mass spectrometry. In order to satisfy the MALDI compatibility requirements, the use of reagents which inhibit or interfere with ionization of the analytes and slow down crystallization of the sample with matrix materials had to be avoided in our preparations of CE electrolyte solutions. Previously, we have successfully used 1% acetic acid solution as running electrolyte for CE separations of tryptic digests[23]. However, the use of this dilute electrolyte brings several disadvantages, namely instability of the system and low sensitivity in detection by UV. The solution has a low ionic strength, which results in extremely low currents in the capillary during electrophoresis. Our experience showed that if longer (> 40 cm) capillaries are used, only sub-μamp currents are generated and higher separation voltages must be applied, often causing instability or failure of the CE power supply. Because the ionic strength of the sample electrolyte must always be lower than that of the running electrolyte to ensure optimal separation of the sample components[24], hydrodynamic introduction of the sample into the capillary causes the current to drop even lower. Moreover, UV absorption of 1% acetic acid solution in the desired wavelength range (200-260 nm) is high relative to what it is for some other common buffers used in CE-UV analyses, such as phosphate and borate buffers[25]. Hence, one is limited to using relatively short capillaries and applying small volumes of highly concentrated samples to the capillaries. Because glycopeptide-containing samples are already "diluted" in terms of microheterogeneity in glycosylation, electrolyte systems which would allow for injection of larger sample volumes without compromising separation and resolution of the sample components had to be investigated.

cdrqr**vpeca nlmtvapitN atmdllsgk**w fyigsafr**np eyNk**saraiq
aaffyleprh aedklitrey qtiedk**cvyN csfik**iyr**qN gtlsk**vesdr
ehfvdlllsk hfr**tfmlaas wNgtk**nvgvs fyadkpevtq eqkkefldvi
kcigiqesei iytdekkdac gplekqheee rkketeas

Figure 2.1. Amino acid sequence of bovine α_1-acid glycoprotein with predicted *N*-glycosylated tryptic fragments. Segments of the sequence in bold were identified as tryptic peptides with possible glycosylation sites.

2.5.2 Sample fractionation by reversed-phase liquid chromatography

The amino acid sequence of bovine α_1-acid glycoprotein was examined *in silico* in order to establish potential *N*-glycosylation sites in the protein and to predict the masses of possible tryptic fragments, which would contain these sites (Figure 2.1). Five asparagine residues were given high scores by the NetGlyc algorithm as potential glycosylation sites. Following *in silico* digestion of the protein with trypsin (no missed cleavage allowed), five tryptic peptides were predicted to bear *N*-linked glycans (Table 2.1). The protein was then digested with trypsin and a portion of the digest was treated with PNGase F to remove the glycans from the peptides. A portion of the crude tryptic digest as well as the PNGase F-treated digest were separated into 30 fractions by reverse-phase chromatography. This was done in order to isolate fractions containing glycopeptide pools, and also to enhance detection of glycopeptides by minimizing ion suppression effects inherent to complex peptide mixtures. In addition, the separation served as a desalting step for subsequent analysis by capillary electrophoresis.

Table 2.1. Predicted *N*-glycosylated tryptic peptides of bovine α_1-acid glycoprotein and indication of their observation by off-line LC MALDI-TOF-MS.

Tryptic Fragment	Sequence	$[M+H]^+$	pI	Observed
T_{89-95}	qNgtlsk	747.40	8.75	-
T_{39-44}	npeyNk	764.36	6.00	+
T_{77-85}	cvyNcsfik	1076.49	8.05	-
$T_{114-125}$	tfmlaaswNgtk	1326.63	8.41	-
T_{6-29}	vpecanlmtvapitNatmdllsgk	2489.25	4.37	-
$^*T_{89-95}$	qDgtlsk	748.38	5.84	+
$^*T_{39-44}$	npeyDk	765.34	4.37	+
$^*T_{77-85}$	cvyDcsfik	1077.48	5.82	+
$^*T_{114-125}$	tfmlaaswDgtk	1327.61	5.50	+
$^*T_{6-29}$	vpecanlmtvapitDatmdllsgk	2490.23	4.03	

* Predicted tryptic glycopeptides with Asn residues replaced by Asp

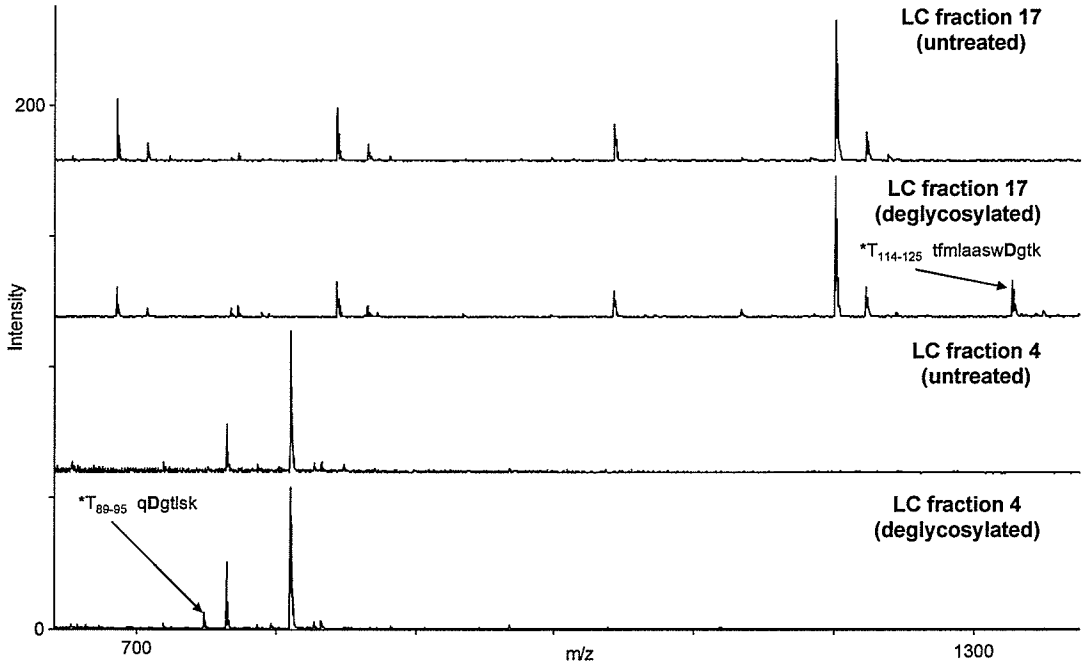

Figure 2.2. Mass spectra of untreated and deglycosylated LC fractions. These spectra only differ by the appearance of the predicted deglycosylated tryptic fragments and disappearance of several peaks at higher masses in deglycosylated fractions. Refer to Table 2.1 for *m/z* assignment.

MALDI-TOF mass spectra were acquired for all fractions in the three sets (the untreated, deglycosylated and asialo sets). Comparison of the mass spectra within each subset allowed for determination of the *N*-glycosylation sites in the protein. Good reproducibility in separations by the reversed-phase chromatography method employed, as illustrated by the mass spectra of Figure 2.2, allowed for a higher degree of accuracy in the assignment of fractions based on their glycopeptide content. As previous data suggest that attached glycans do not greatly influence the elution time of peptides under reversed-phase chromatography conditions[26], it was possible to make an indirect assignment of the crude tryptic digest fractions containing *N*-linked glycopeptides. The mass spectrum of a tryptic digest LC fraction was compared to that of a PNGase F-treated digest fraction. If a predicted tryptic fragment with an increased mass of 1 Da, indicative of transformation of asparagine into aspartic acid, was observed in a deglycosylated digest fraction and if none were seen in the crude tryptic digest fraction, it implied that the glycans were successfully removed from the peptide by the endoglycosidase (Figure 2.3). The absence of several peaks at higher masses in the deglycosylated fractions – the peaks which are observed in the untreated sample fractions, indicates that those peaks correspond to glycopeptides. Four LC fractions of the untreated tryptic digest were thus found to contain *N*-linked glycopeptides.

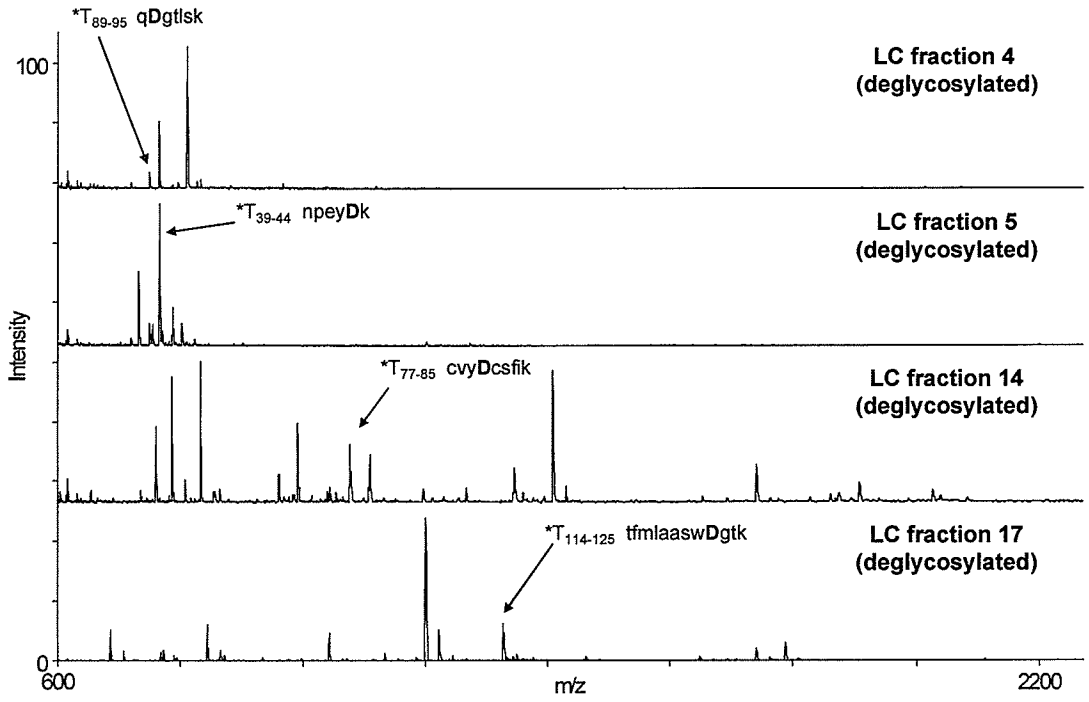

Figure 2.3. MALDI mass spectra of LC fractions containing deglycosylated tryptic peptides of bovine α_1-acid glycoprotein with predicted *N*-glycosylation sites. Refer to Table 2.1 for *m/z* assignment.

Glycopeptide-containing fractions from the asialo sample also showed peaks at higher masses, although fewer, with a general group shift towards the lower mass, as shown in Figure 2.4. This indicated that desialylation with H_2SO_4 was successful. Thus, glycopeptides observed within each group in the "untreated" and "asialo" fractions are related to the deglycosylated tryptic peptide seen in the "deglycosylated" fraction. This was confirmed by MS/MS experiments, which showed the absence or the presence of sialic acid, as well as the identity of the peptide bearing the glycans within each fraction (Figure 2.5).

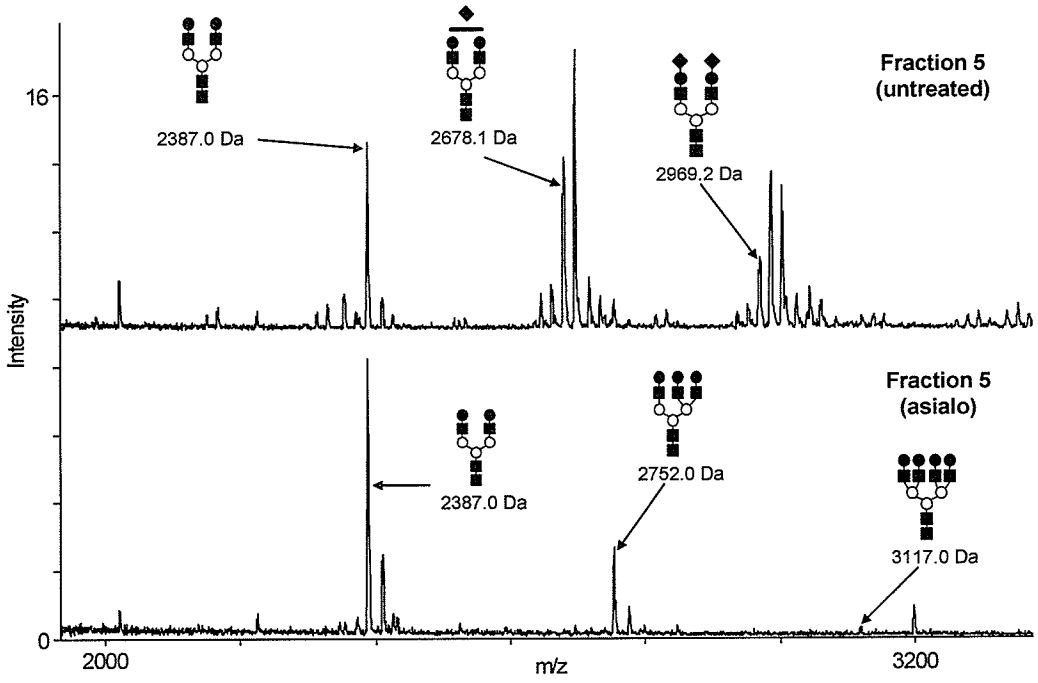

Figure 2.4. Comparison of sections of MALDI spectra showing the glycopeptide portion of untreated LC fraction 5 (top), and desialylated LC fraction 5 (bottom). Refer to Tables 2.1 and 2.2 for *m/z* assignments.

Unambiguous assignment of the peptide-bearing glycans in a given LC fraction was made possible by the observation of two high-abundance peaks corresponding to $[Peptide + H]^+$ and $[Peptide + 204]^+$ ions in the tandem mass spectra of the glycopeptides[26], as seen in Figure 2.5 A-C. The latter mass is attributed to an ion composed of a peptide, a proton and a single GlcNAc residue. The region between the parent ion and $[Peptide + 204]^+$ contained peaks resulting from successive losses of individual monosaccharide units. This information was used to characterize glycans in terms of composition and sialylation. Bi- and triantennary complex structures were found to be present in all four LC glycopeptide-containing fractions.

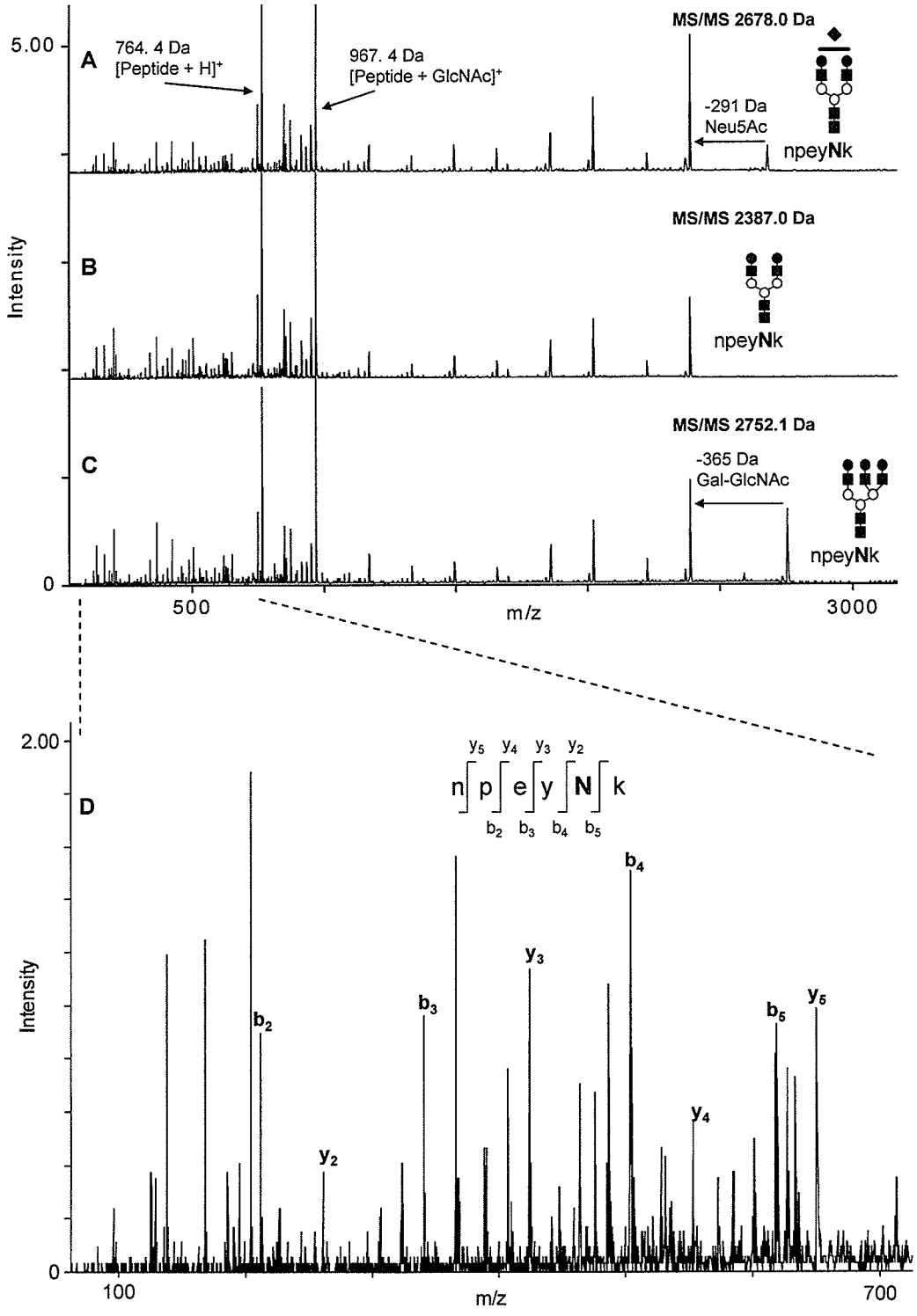

Figure 2.5. MALDI-MS/MS spectra of $[M+H]^+$ ions of glycoforms of npeyNk peptide from bovine α_1-acid (A, B, C). D: expansion of peptide fragment portion, common to all these spectra.

Masses corresponding to tetrasialo structures were also observed, however these were present in very low abundance although their identity was confirmed by MS/MS (not shown). The region downstream of the [Peptide + H]$^+$ contains information pertaining to the amino acid composition in a peptide in a form of characteristic b and y peptide cleavage fragments, which can be used to confirm identity of the peptide, is shown in Figure 2.5 D.

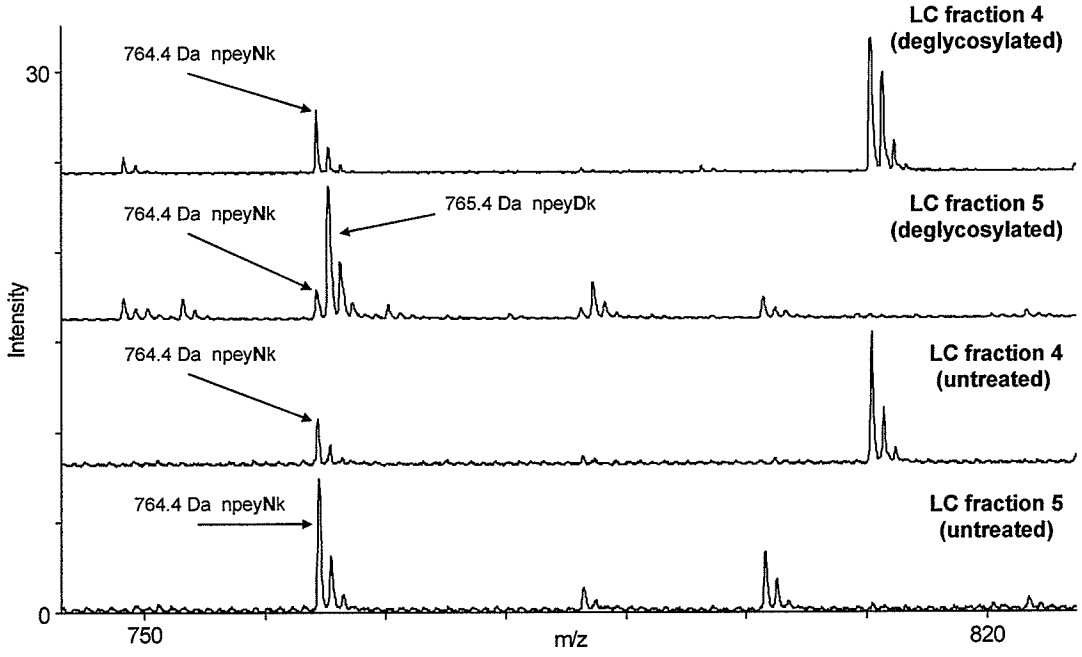

Figure 2.6. Portions of MALDI spectra of untreated and deglycosylated LC fractions 4 and 5, showing two different forms of tryptic peptide npeyNk from α_1-acid glycoprotein.

Table 2.1 provides a summary of the *N*-glycosylation sites determined in the bovine α_1-acid glycoprotein. Predicted tryptic peptides as well as their deglycosylated analogues are listed in the table. One predicted tryptic *N*-glycosylated fragment was not

observed, nor was its deglycosylated form. This could result from omitting the reduction/alkylation step in the tryptic digestion procedure, leading to formation of Cys-bridged tryptic products. The chicken homologue of the protein was found to contain four Cys forming two disulfide bonds[27]. The unobserved glycopeptide does contain a Cys residue, which may have been involved in a disulfide bond. We decided not to investigate this matter further, since the observation of the other four provides sufficient amount of working material for establishing a general methodology for separating the glycopeptides under investigation. Interestingly, one of the predicted *N*-glycosylation sites appears to be partially glycosylated, as the peptide with unmodified Asn was observed in both the untreated and deglycosylated fractions (Figure 2.6). The deglycosylated form was seen only in the PNGase-treated fraction. The identity of these peptides was confirmed by MS/MS analysis (data not shown). This result is consistent with the observation of partial glycosylation at an equivalent site in chicken α_1-acid glycoprotein[27].

An interesting feature in the mass spectra of the untreated sample fractions is the clustering of glycopeptide ions, in which peaks separated by 16 Da appear within each group (Figure 2.7 A). The mass difference between the major glycoform groups corresponds to the residual mass of a sialic acid. This is absent from the mass spectra of asialo samples, where only the sodium adduct ions are seen together with the $[M+H]^+$ ions (not shown). This difference of 16 Da is attributed to the presence of both N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc) sialic acids in the glycopeptides. Neu5Ac has a residual mass of 291 Da, whereas that of Neu5Gc is 307 Da. The two structures differ by the presence or absence of a hydroxyl group on the N-

acetyl group of the sugar. This becomes evident when comparing the MS/MS spectra of $[M + H]^+$ peaks within each cluster (Figure 2.7 B-D).

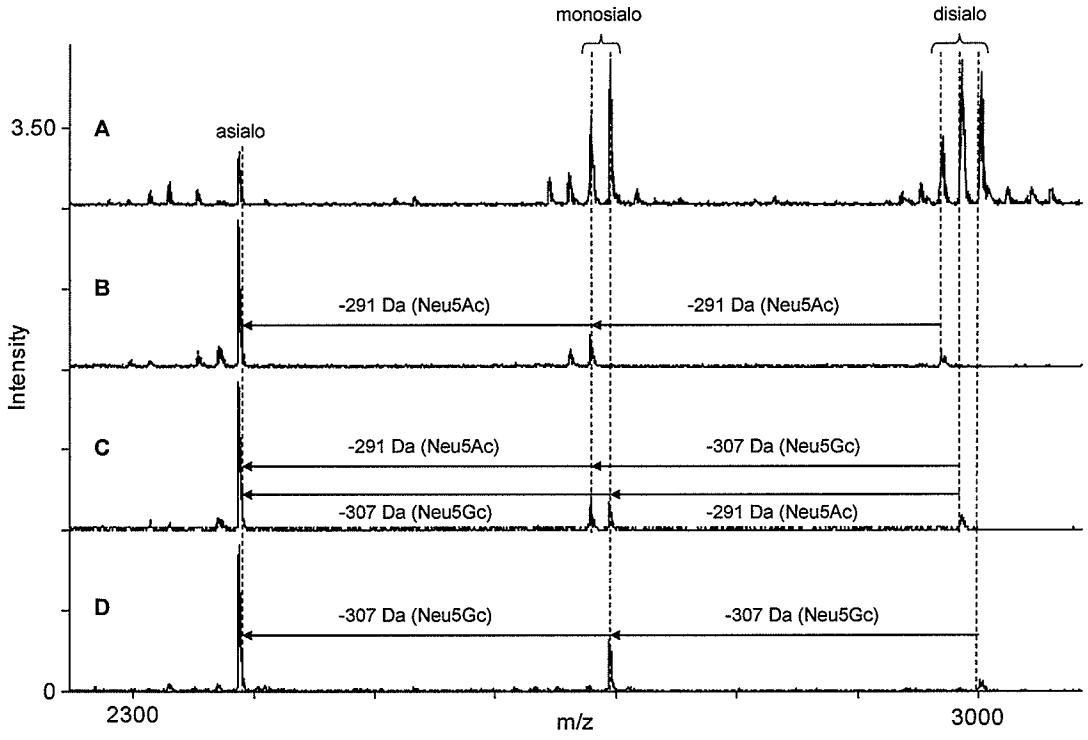

Figure 2.7. A: MALDI mass spectra of glycoforms of npeyNk; B-D: tandem mass spectra of $[M+H]^+$ ions of different forms of disialo species.

One may also determine the number of sialic acids in a particular glycopeptide by counting the number of peaks within a given cluster. For example, if a doublet separated by 16 Da is observed, then the glycan bears only a single sialic acid residue. If the next cluster is separated by a mass roughly equivalent to that of sialic acid, this cluster will contain three peaks. This may be generalized by stating that *n* number of sialic acids in a glycan will result in $n + 1$ peaks in the peak cluster. The observation of these multiplets

of peaks in a given sample implies presence of Neu5Gc and Neu5Ac and allows to determine the number of sialic acids present on the glycan. This strategy may not be employed for analysis of sialylated glycans in human proteins, since humans lack the active form of the enzyme for converting Neu5Ac to Neu5Gc, however occurrences of such patterns may be indicative of a pathological state in humans or an increase in incorporation of exogenous Neu5Gc[28-30]. All sialylated glycans of a single composition in human samples are therefore expected to be represented by a single peak, and appearance of the multiplet phenomenon would be indicative of some abnormality. Using the above approach, we were able to make a crude assignment of the degree of sialylation of a glycan on a particular glycopeptide seen in the mass spectrum.

2.5.3 Isolation of glycopeptides by capillary electrophoresis

The three sets of LC fractions were further subjected to analysis by CE to investigate the impact of glycosylation and presence of sialic acid residues on electrophoretic migration of the peptides in the capillary. Our choice of ammonium acetate as a running electrolyte was based on volatility and on its significantly higher conductivity and lower absorbance at 214 nm then 1% acetic acid solution. This made possible the use of longer capillaries, and thus the ability to inject larger volumes of samples without pre-concentration. Moreover, the use of 1% acetic acid solution as sample electrolyte allowed not only to maintain the pH of the sample slightly above the pI of sialic acids (~2.6) to ensure that sialylated glycopeptides carry a negative charge, but it also resulted in efficient stacking and resolution of peptides in the samples analyzed.

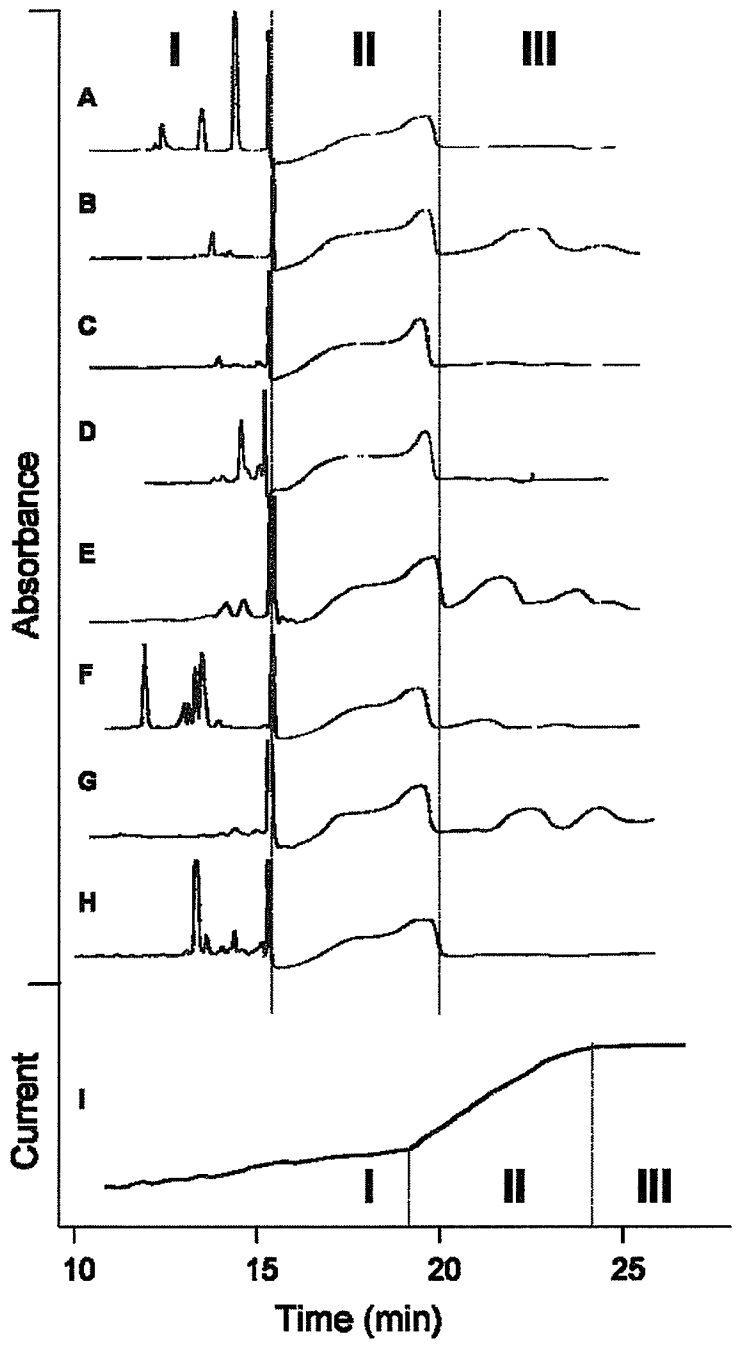

Figure 2.8. A: CE-UV trace obtained for a standard mixture of peptides; B-H: CE-UV traces for different LC fractions: untreated (B, E, F, G), deglycosylated and desialylated (C and D), and a glycopeptide-free fraction (H). I: Current trace obtained during these experiments. Sections labeled I-III on the electropherograms and the current trace correspond to peptide/non-sialylated glycopeptide (I), sample plug (II), and sialylated glycopeptide (III) containing region.

All electropherograms obtained, including a sample electrolyte blank, contained a "sample plug" absorbance feature – a broad peak as a result of the sample plug migrating past the detector window, as shown in Figure 2.8. This is attributed to the difference in UV absorption and ionic strength of the sample electrolyte relative to the running buffer. Due to significantly lower conductivity in the sample electrolyte, the application of CE separation voltage results in very high field strength through the sample plug region. Because the sample is at pH 2.8, most peptides are expected to have a net positive charge and migrate towards the cathode with rapid stacking at the leading interface between the two electrolytes. Further separation will depend on the mobility of a given peptide in the running electrolyte. The current through the capillary is lowered upon injection of the 1% acetic acid sample plug, as expected. Elution of the sample plug can be monitored by observing the evolution of the current trace during electrophoresis. A rapid increase in the current through the capillary marks the beginning of elution of the sample plug (Figure 2.8 I). Therefore, the mixture components lagging the sample plug in their elution profile may easily be separated from the rest of the mixture by stopping the run once the sample plug leaves the capillary and eluting components which follow with pressure.

Injection of a 4 peptide standard mixture consisting of angiotensin II (1045.5 Da), substance P (1346.7 Da), bombesin (1618.8 Da), and $ACTH_{1-17}$ (2092.1 Da) at ~10^{-6} M each resulted in good resolution of the peptides in the region of the electropherogram preceding the sample plug feature, as seen in Figure 2.8 A. Broad peaks following the sample plug were observed in the electropherograms of all four untreated fractions (Figure 8 B, E, F, G). We also analyzed several LC fractions of the untreated sample that were not found to contain glycopeptides and no peaks following the sample plug were

observed in the electropherograms (e.g., Figure 2.8 H), suggesting that these peaks were due to glycopeptides. Electropherograms of the corresponding deglycosylated and asialo fractions (Figure 2.8 C and D) that contained glycopeptides also lacked these peaks, suggesting that these were due to sialylated glycopeptides. This implies that presence of negatively charged sialic acid residues on the glycans significantly affects migration of the glycopeptides under these conditions: instead of migrating together with other peptides at the leading electrolyte interface, these appear to move towards the opposite end of the sample plug, "diffusing" into the running electrolyte towards the anode. This reverse migration is apparently compensated for by the electroosmotic flow generated within the capillary, which results in a somewhat retarded net forward migration. Ultimately, sialylated glycopeptides are separated from the other peptides in the sample, including asialo-peptides, by the length of the sample plug. It is also important to point out that the profile of the sample plug feature in the electropherograms of untreated, asialo, and deglycosylated samples remained relatively invariable relative to a blank, suggesting that all analytes are charged and participate in electrophoretic migration.

In order to evaluate this hypothesis, glycopeptide-containing LC fractions were each separated into two fractions by CE. Sample coming off the capillary up until the end of elution of the sample plug (as indicated by leveling off of the capillary current upon its sharp increase from the starting value) was collected as a first "peptide" fraction. The second fraction was collected by a subsequent hydrodynamic flushing of the capillary with running electrolyte equivalent to one volume of the capillary. These were collected directly onto a MALDI target using the BD^{TM} MALDI sample concentrator device and analyzed by MALDI-TOF mass spectrometry. We found that only the second

CE fractions obtained from untreated samples contained peaks at higher masses – the glycopeptide-containing region where several glycopeptides were identified prior to CE separation (Figure 2.9). MS/MS analyses of these higher mass peaks confirmed their identity as glycopeptides. Second CE fractions of deglycosylated and asialo samples did not show any peaks in that region of the mass spectrum and were essentially blank.

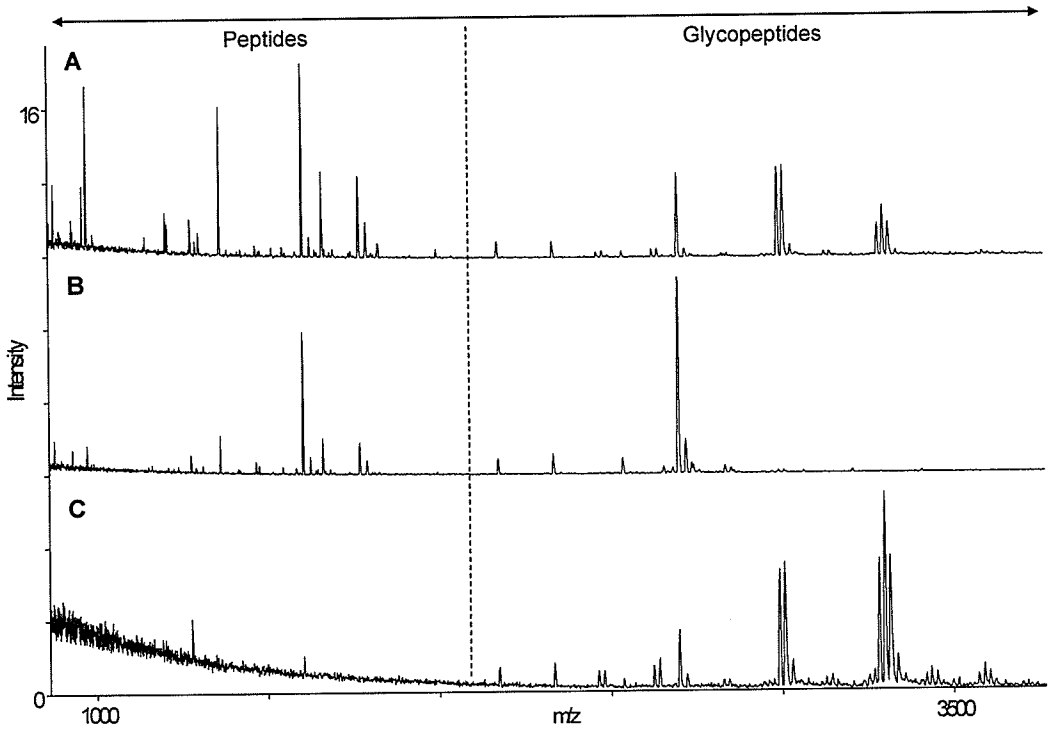

Figure 2.9. MALDI mass spectra of a LC fraction 14 of a tryptic digest of α_1-acid glycoprotein. A: complete untreated fraction; B: complete desialylated fraction; C: sialylated glycopeptides isolated by CE from complete sialylated fraction.

Mass spectra of the untreated "glycopeptide" CE fractions show peaks corresponding to ions with asialo glycans. As no compounds were detected in the "glycopeptide" fractions of asialo samples, we believe that these ions are likely generated

in-source and that all glycopeptides isolated in the sialo "glycopeptide" CE fraction are sialylated. This further implies that presence of glycan structures alone does not permit for purification of the total glycopeptide pool: only sialylated glycopeptides can be completely isolated and this separation is based primarily on charge of glycopeptides.

Large broad peaks observed in the "glycopeptide" regions of the electropherograms pertaining to untreated samples therefore likely correspond to the net charge states, and thus the number of sialic acid residues on the glycopeptides, independent of the degree of oligosaccharide branching. The migration order of these peaks is therefore directly proportional to the number of sialic acids residues found on the glycans of a given glycopeptide. This feature is also observed in high-pH anion exchange chromatography and in-gel isoelectric focusing, techniques often used to separate glycoprotein oligosaccharides[31]. The fact that only two main peaks were observed in that region in all four samples as seen in Figure 2.8, each representing glycan pools specific for each glycosylation site on the protein, indicates that the majority of the sialic acid-containing glycans on the protein are in mono- and disialylated forms. In addition to the typical sialylated glycans, we also identified several structures bearing disialyl structures on the same branch. Glycan compositions are listed in Table 2.2. Although mass spectra showed the presence of tri- and tetrasialylated glycopeptides, these were in very low abundance and not clearly observed in the CE UV traces. Nevertheless, the fractions collected by pressure did not limit themselves to mono- and disialo compounds.

Table 2.2: Composition of glycopeptides found in four different LC fractions of tryptic digests of α_1-acid glycoprotein isolated by CE.

LC Fraction	Tryptic Peptide	Glycopeptide $[M+H]^+$ *m/z*	Glycan Composition
4	qNgtlsk	2661.1[a]	$Neu_1Gal_2GlcNAc_2Man_3GlcNAc_2$
		2952.1[a]	$Neu_2Gal_2GlcNAc_2Man_3GlcNAc_2$
		3026.2[b]	$Neu_1Gal_3GlcNAc_3Man_3GlcNAc_2$
		3317.3[b]	$Neu_2Gal_3GlcNAc_3Man_3GlcNAc_2$
5	npeyNk	2516.1[a]	$Neu_1Gal_1GlcNAc_2Man_3GlcNAc_2$
		2678.1[a]	$Neu_1Gal_2GlcNAc_2Man_3GlcNAc_2$
		2969.1[a]	$Neu_2Gal_2GlcNAc_2Man_3GlcNAc_2$
		3043.2[b]	$Neu_1Gal_3GlcNAc_3Man_3GlcNAc_2$
		3260.2[a]	$Neu_3Gal_2GlcNAc_2Man_3GlcNAc_2$
		3334.3[a]	$Neu_2Gal_3GlcNAc_3Man_3GlcNAc_2$
		3625.3[b]	$Neu_3Gal_3GlcNAc_3Man_3GlcNAc_2$
14	cvyNcsfik	2988.1[a]	$Neu_1Gal_2GlcNAc_2Man_3GlcNAc_2$
		3279.2[a]	$Neu_2Gal_2GlcNAc_2Man_3GlcNAc_2$
		3570.3[b]	$Neu_3Gal_2GlcNAc_2Man_3GlcNAc_2$
17	tfmlaaswNgtk	3240.4[a]	$Neu_1Gal_2GlcNAc_2Man_3GlcNAc_2$
		3531.5[b]	$Neu_2Gal_2GlcNAc_2Man_3GlcNAc_2$

Neu: Neu5Ac sialic acid; Gal: galactose; GlcNAc: N-Acetyl glucosamine; Man: mannose.
[a]: structures determined by MS/MS.
[b]: proposed structures based on glycan mass

Sialylated glycopeptides were successfully separated from the rest of the peptides in a mixture as a discrete group in one simple step for each of the glycopeptide-containing LC fractions. An additional advantage of our method is that relatively large volumes of the samples may be injected onto the capillary. We were able to inject sample volumes up to 40% of the total capillary volume and, although the separation of the peptides at the leading end of the sample plug was greatly diminished, sialylated glycopeptides at the other end were recovered without apparent loss. This can therefore

be used as a preparative method for pre-concentrating the sialylated glycopeptides contained within a given sample for further structural studies by MALDI-MS.

2.6 Conclusions

This study emphasizes the advantage of using two-dimensional separations when characterizing complex biological mixtures. The method demonstrates the benefit of using liquid chromatography as the first dimension in glycosylation site-specific glycan analysis. Although we have shown that it is possible to efficiently separate sialylated glycopeptides from a desalted tryptic mixture, mass spectra of these purified glycopeptide fractions are still complicated and may be difficult to analyze. Fractionation of the sample prior to electrophoresis ensures that each isolated glycopeptide pool originates from a particular peptide, thus allowing for easy assessment of the glycans attached to a specific site on a protein. Additional resolution of peptides found in a given LC fraction by capillary electrophoresis and demonstrated ability of convenient further fractionation of the sample in capillary electrophoresis based separations, prove the value of capillary electrophoresis as a practical second dimension for separations of complex biological mixtures for further analysis by MALDI mass spectrometry. It would also be possible and useful to use CE directly as a sole dimension to separate sialylated glycopeptides from the whole tryptic digest of a glycoprotein in a single step and subsequent sensitive detection of these peptides by MALDI-TOF mass spectrometry. This can be conducted with minimal sample loss due to handling, as the fractions are deposited directly onto a MALDI target.

2.7 Acknowledgements

The authors wish to acknowledge Drs. K. G. Standing and W. Ens for the use of the MALDI-QqTOF instrument and Dr. Kevin Chen at BD™ Biosciences for providing MALDI sample concentrator devices. The Natural Sciences and Engineering Council of Canada (NSERC), the Canada Research Chairs program (CRC) and the Canadian Foundation for Innovation (CFI) are acknowledged for funding. The authors would also like to thank Drs. Erika Lattova, Gro Thorne-Tjomsland and Mark McComb for helpful discussions.

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Chapter 3:

Development of an LC-based method for isolating glycopeptides with cellulose material for glycoproteomic and/or glycomic studies.

3.1 Authors contributions

The work described in this section was initiated in the summer of 2008 at the Institute of Biological Chemistry of Academia Sinica (Taiwan) as part of Summer Research in Taiwan program put into operation by the Natural Sciences and Engineering Council (NSERC) Canada and National Science Council (Taiwan). Sergei I. Snovida did all preliminary work in the lab of Dr. Kay-Hooi Khoo at Academia Sinica. The remainder of the project was conducted in the lab of Dr. Hélène Perreault at the University of Manitoba by Sergei I. Snovida with assistance by Edward Bodnar. All ESI LTQ-OrbiTrap data was acquired and processed by Drs. Rosa Viner and Julian Saba (Thermo Fisher Scientific, San Jose, CA, USA). Sergei I. Snovida designed and conducted all experiments in this study and is responsible for preparation of the manuscript, including production of all associated figures.

Manuscript pertaining to the work presented in this section of the thesis is currently in preparation and additional experiments are being conducted at the time of writing.

3.2 Abstract

In this report we describe an on-column method for glycopeptide enrichment with cellulose as solid-phase extraction material. The method was developed using tryptic digests of several standard glycoproteins and validated with more complex standard protein digest mixtures. Glycopeptides of different masses containing neutral and acidic glycoforms of both *N*- and *O*-linked sugars were obtained by this method in good yield. Upon isolation, glycopeptides may be subjected to further glycoproteomic and glycomic workflows for the purpose of identifying glycoproteins present in the sample, their glycosylation sites, as well as their global and site-specific glycosylation profiles at the glycopeptide level. Detailed structural analysis of glycoforms may then be performed at the glycan level upon chemical or enzymatic release of the oligosaccharides. Aiming at replacing or complementing lectin-based purification methods, this technique is extremely simple, cost-effective, and efficient.

3.3 Introduction

Protein glycosylation is an area of active research in the realm of biological sciences[1]. It is a widely accepted notion that oligosaccharide moieties are important modifications that play a significant role in many biochemical processes ranging from fine-tuning of protein folding to receptor site recognition events in some key signaling pathways[2,3]. Much of the current research in the field of glycoproteomics is focused on development of methods, which would lead to sensitive detection of glycans and various glycoconjugates, with particular emphasis on accurate quantitative analysis and detailed structural characterization[4-6].

In terms of sensitivity in the analysis of biomolecules, mass spectrometry (MS) is one of the most useful analytical techniques available today. Both ESI and MALDI-based methods[7-9] are employed in glycoproteomics, often offering complementary sets of data. This information is used to provide comprehensive characterization of sample analytes. Samples from different biological sources are analyzed routinely at the low femtomole levels and the limits of detection are constantly improving with each new generation of MS instruments. A good example of the most recent leap in evolution of MS instruments is the introduction of OrbiTrap™ instruments[10] by Thermo Fisher Scientific, which offer substantial improvements in both mass accuracy and sensitivity. Coupled with various modes of separation techniques, MS in general provides a very powerful platform for studying glycosylation of proteins[11].

Successful application of MS in glycoproteomics greatly depends on the workflows adopted to address specific questions relating to a particular sample type. Thus, much attention must be devoted to development of sample preparation techniques that would

ensure utilization of an instrument to its full unique ability in order to extract all potentially accessible information from this sample. Targeted enrichment of sample constituents is one such procedure. In principle, this kind of purification step greatly reduces the complexity of the overall sample matrix, thus facilitating more sensitive and accurate analysis of compounds of interest.

With respect to sample purification in glycoproteomics, it is possible to carry out these procedures at the protein, peptide, and/or glycan levels, depending on the stage in the overall workflow or on the nature of information sought. If one is required to identify all sample glycoproteins, including their glycosylation sites, initial enrichment at protein or peptide level is required. Purification of glycoproteins may be performed to selectively isolate a certain glycoprotein, as well as entire glycoproteomes, on the basis of specific glycan structures. These methods are usually based on lectin affinity interactions and numerous reports of their applications are available in the literature[12-14]. Although potentially useful for purifications at all three levels of glycoproteomic analysis, these techniques are relatively expensive and are generally limited by poor yields. Alternatively, purification is done at the glycopeptide level without extracting the initial glycoprotein extraction. These methods typically invoke the use of various hydrophilic interaction liquid chromatography (HILIC) techniques[15-20]. This approach offers several advantages in terms of simplifying workflows. All glycoproteins present in a sample may be potentially identified through database sequence matching using amino acid sequences of relevant glycopeptides alone. In addition to this, information on location of glycosylation sites, as well as their site-specific glycoform distribution, is preserved.

Among several promising stationary phase materials available for glycopeptide isolation, cellulose is exceptionally well suited, owing to its wide availability, low cost, respectable efficiency, and the overall ease of use in purification protocols[15,21]. In this report we further investigate efficiency of cellulose in an on-column set-up for glycopeptide enrichment. Examples of use of Thermo LTQ OrbiTrap instrument in analysis of glycopeptides are also presented in this article.

3.4 Experimental

3.4.1 Materials and reagents

Human and bovine α_1-acid glycoprotein, bovine fetuin, SigmaCell Type 20 cellulose (20 μm average particle size), iodoacetamide (IAA), and dithiothreitol (DTT) were purchased from Sigma (St. Louis, MO, USA). A 10 protein standard mixture consisting of lysozyme (~0.12 mg), GAPDH (~0.12 mg), β-casein (~0.12 mg), cytochrome-C (0.12 mg), BSA (~0.12 mg), ovalbumin (~0.12 mg), carbonic anhydrase (~0.12 mg), transferrin (~0.12 mg), α-lactalbumin (~0.06 mg), and apo-myoglobin (~0.02 mg) was obtained from Pierce (Rockford, IL, USA). PNGase F endoglycosidase and proteomics-grade trypsin were bought from ProZyme (San Leandro, CA, USA). C_{18} bulk reversed-phase chromatographic material (15 μm average size, 300 Å pore size) was purchased from J.T. Baker (Phillipsburg, NJ, USA). Cellulose solid-phase extraction cartridges were purchased from Takara Bio Inc. (Otsu Shiga, Japan). All solvents were HPLC-grade and were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Milli-Q water was used in all experiments. Frits and 8 mL solid phase extraction tubes were obtained from Grace Discovery Sciences (Deerfield, IL, USA). Dialysis membranes

(6000-8000 Da molecular weight cutoff) were purchased from Spectrum Laboratories (Rancho Dominguez, CA, USA).

3.4.2 Sample preparation

Tryptic digests of individual glycoproteins were prepared by dissolving about 1 mg of a given protein in 200 μL of 25 mM ammonium bicarbonate buffer (pH 7.8) in a 1.5 mL microcentrifuge tube. Upon complete dissolution of the protein, 5 μL of trypsin solution (as provided by the manufacturer) were added. The mixture was vortexed and allowed to incubate for 24 hours at 37 °C.

In some cases, as stated in the Results and Discussion section, proteins were reduced and alkylated prior to digestion with trypsin according to a generally adopted reduction/alkylation procedure. Briefly, proteins were dissolved in 200 μL of 5 mM dithiothreitol (DTT) solution (in 25 mM ammonium bicarbonate). After one hour of incubation at 37 °C, 50 μL of 20 mM iodoacetamide (IAA) solution were added and the mixture was left standing in the dark for 1 hour. The mixture was then dialyzed 3 times against Milli-Q water (2 liters replaced every 10 hours). Recovered proteins were lyophilized and digested with trypsin according to the procedure stated above. After digestion with trypsin, samples were lyophilized and re-suspended in 500 μL of 0.1% trifluoroacetic acid in water.

3.4.3 Cellulose column preparation

Preliminary work involved use of commercially available cellulose solid-phase extraction cartridges (Takara Bio Inc.). In-house glycopeptide extraction columns were

constructed according to the illustration in Figure 3.1. Bulk cellulose (~10 grams) was washed 3 times with 50 mL of water and 3 times with 50 mL of MeOH, successively, in order to remove any soluble impurities. The liquid portion of the resulting suspension was removed by centrifugation each time. Cellulose material was then loaded into an 8 mL solid-phase extraction tube plugged with a frit as a suspension in MeOH to the net volume of ~2 cm^3 (after settling). C_{18} material was then added as a suspension in MeOH to a final column packing volume of ~2.5 cm^3. The packing material was then gently compressed with a second (top) frit, which was left in the column to prevent any disturbance of the packing material during use.

3.4.4 Extraction of glycopeptides

The cellulose column was conditioned by washing with 10 mL of 0.1% TFA in water solution (2 mL at a time). Protein digest samples (100 -500 μL) were loaded onto the column running under ambient conditions without vacuum assistance. The column was then washed with 1 mL of 0.1% TFA in water twice to desalt the sample and to ensure that all sample peptides were bound to the C_{18} material. Peptides were eluted with 5 mL of 0.1 % TFA in 1:9 water/MeOH solution as a "peptide" fraction. Glycopeptides were eluted with 5 mL of 0.1% TFA in 7:3 water/MeOH solution as a "glycopeptides" fraction. Both fractions were lyophilized and re-suspended in 0.1% TFA in water solution to volumes equal to those of the initially loaded samples.

3.4.5 Liquid Chromatography and Mass spectrometry

All preliminary work was done on an ABI 4700 MALDI TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) operated in both linear and reflective positive ion modes. Additional MALDI MS experiments were performed on the University of Manitoba/Sciex MALDI-QqTOF instrument (Department of Physics, University of Manitoba, Canada; PE Sciex, Mississauga, ON, Canada). ESI-MS experiments were performed on Thermo LTQ-OrbiTrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) in the positive ion mode.

Experiments were performed on an LTQ Orbitrap XL™ ETD mass spectrometer (Thermo Scientific, Bremen, Germany) with a nano-ESI source that was coupled to a Surveyor™ MS Pump with a flow splitter. For LC-MS/MS analysis, protein digests were separated on a PicoFrit™ ProteoPep™ 2 , 5 or 10 cm x 75 μm i.d. column (New Objective, Woburn, MA) or Thermo Hypercarb 5 cm x 180 μm i.d. column (Thermo Fisher Scientific, Waltham, MA). Gradient elution was performed from 5-45% acetonitrile in 0.1% formic acid over 30 min at a flow rate of ~300 nL/min. The Orbitrap mass spectrometer was operated at 60,000 or 100,000 resolution (FWHM at *m/z* 400) in full MS, with a maximum ion injection time of 300 msec, and an automatic gain control (AGC) setting of 500,000 ions. AGC was set to 10,000 ions for MS/MS analysis in the ion trap and to 100,000 ions for the MS/MS analysis in the Orbitrap. HCD normalized collision energy was set to 45% and fragment ions were detected in the Orbitrap at a resolution of 7500 (FWHM at *m/z* 400) using 1 microscan, with a maximum injection time of 300 msec. For ion trap ETD spectra isolation of 3 amu, 3 microscans with a maximum injection time of 150 msec were used. ETD reactions were performed based on

charge state with the anion AGC target set at 200,000. LC-MS/MS acquisition methods consisted of seven scan events: an MS scan with Orbitrap mass analysis followed by ETD and HCD of the three most abundant precursors. Ion trap MS/MS scans (ETD) were conducted in parallel with the full MS scans. Peptide masses were determined by deconvolution of isotopically resolved spectra using Xtract software (FT programs Thermo Scientific, Bremen, Germany) over the entire time of each LC-MS run.

3.5 Results and discussion

3.5.1 Preliminary work

Most glycopeptide extraction procedures found in the literature are based on the principle of hydrophilic interactions between oligosaccharide portion of a molecule and appropriate solid-phase material[19]. On the basis of previously published work[15,21] on the use of cellulose for isolation of glycopeptides from complex proteolytic digests in solution, we began to examine the use of this inexpensive material in an on-column format. Special emphasis was placed on obtaining high glycopeptide extraction yields with minimal amount of non-glycopeptide material carry-over. Initial work was done using a commercially available cellulose solid-phase extraction cartridge from Takara Bio Inc. marketed specifically for use in glycan purification. All subsequent work was performed using in-house made columns, designed according to Figure 3.1 and described in the Experimental section.

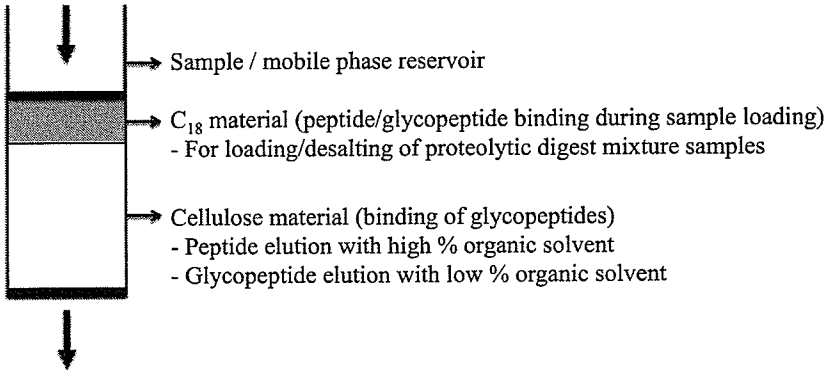

Figure 3.1. Design of a cellulose-based glycopeptide extraction column.

Bovine α_1-acid glycoprotein was chosen as a standard glycoprotein in these experiments due to its high glycan content and multiple glycosylation sites. Glycoforms found on this protein also carry sialic acid, a biologically significant monosaccharide unit. Additionally, this protein is well characterized in terms of its glycosylation patterns and large amounts of reference data are readily available in the literature[22-25]. Upon application of our sample to the column and subjecting the column to "peptide eluting" and "glycopeptides eluting" conditions, as described in the Experimental section, the sample was separated into two fractions, i.e "peptide" and "glycopeptide" fractions. Our hypothesis was that the "peptide" fraction was depleted of glycopeptides, whereas the "glycopeptide" fraction was assumed to contain mostly extracted glycopeptides. The next set of experiments was aimed at testing these hypotheses. Mass spectra of the initial results are presented in Figures 3.2-3.4. Originally, both "peptide" and "glycopeptide" fractions were analyzed in the positive ion reflector mode (Figure 3.2).

Due to the inherently low sensitivity of the reflector mode TOF analysis at the higher *m/z* range, as well as to the occurrence of a large number of glycopeptides with negatively charged glycoforms, none of the expected glycopeptides were observed in either fraction. Several peptides common to both fractions were detected in the "glycopeptide" fraction (Figure 3.2 B), however these were attributed to non-specifically retained peptides. Very broad, low intensity peaks characterized the *m/z* region of anticipated glycopeptide signal occurrence.

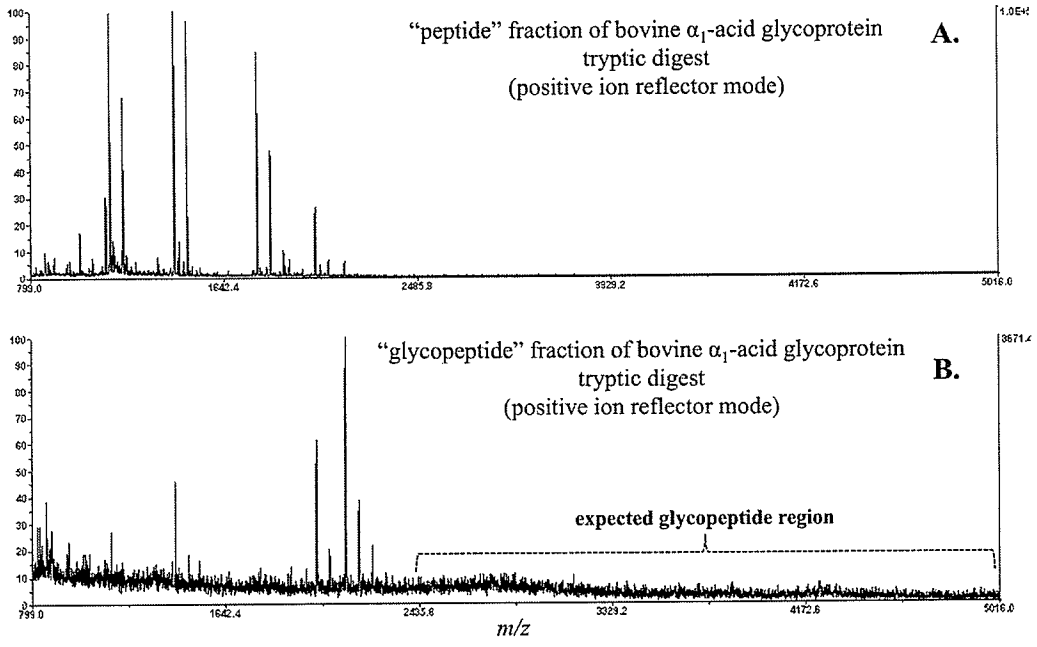

Figure 3.2. Reflector mode MALDI mass spectra of "peptide" (A) and "glycopeptide" (B) fractions of bovine α_1-acid glycoprotein tryptic digest mixture separated on a cellulose column. Spectra were acquired on an ABI 4700 MALDI TOF/TOF instrument in the positive ion mode.

The linear mode MALDI mass spectrum of the "glycopeptide" fraction is presented in Figure 3.3 A. Superior sensitivity of linear mode analysis is evident when

comparing the mass spectra of Figures 3.2 B and 3.3 A: a much higher ion abundance is seen when the sample is analyzed in linear mode. Still, peaks in the *m/z* region where glycopeptide peaks were expected appeared very poorly resolved. In order to remove the potentially present ionization-suppressing, negatively charged sialic acid residues, a portion of this sample fraction was treated with 0.1% TFA solution for 1 hour at 70 °C. The mass spectrum of the acid-treated "glycopeptide" fraction is shown in Figure 3.3 B. Appearance of new, higher peaks at lower *m/z* was a strong indication of the presence of glycopeptides in the "glycopeptide" fraction.

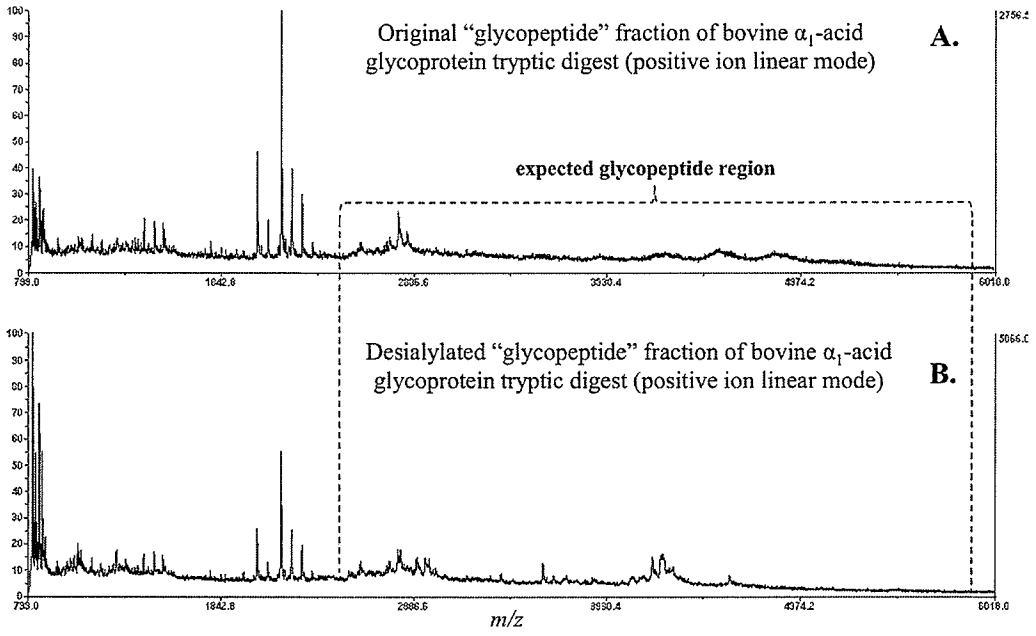

Figure 3.3. Linear mode MALDI mass spectra of "glycopeptide" fraction of bovine α_1-acid glycoprotein tryptic digest mixture isolated on a cellulose column. The desialylated sample (B) was obtained by treating the original sample fraction (A) with 0.1% TFA solution at 70 °C for 1 hour. Spectra were acquired on an ABI 4700 MALDI TOF/TOF instrument in the positive ion mode.

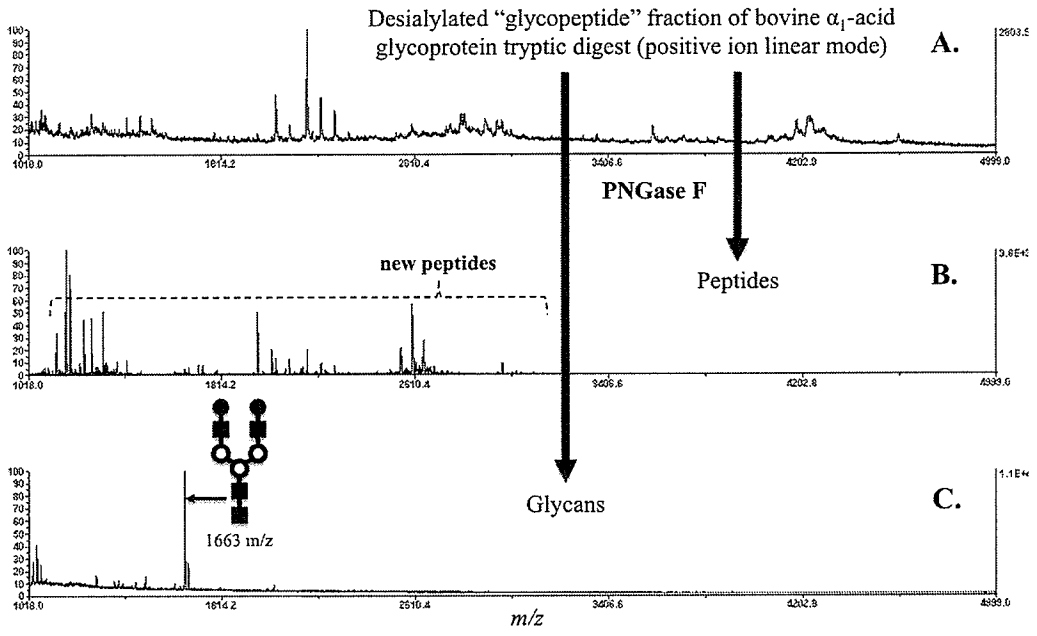

Figure 3.4. Linear mode MALDI mass spectra of "glycopeptide" fraction of bovine α_1-acid glycoprotein tryptic digest mixture isolated on a cellulose column (A); isolated peptides (B) and glycans (C) upon deglycosylation of the original "glycopeptide" fraction with PNGase F. Spectra were acquired on an ABI 4700 MALDI TOF/TOF instrument in the positive ion mode.

The original "glycopeptide" fraction was then treated with PNGase F endoglycosidase and the mixture was subjected to a reversed-phase separation procedure on a C_{18} clean-up column, which is normally used to separate oligosaccharides and peptides. The fraction potentially containing glycans was treated with 0.1% TFA solution to remove sialic acids in order to improve ionization of the native glycans. As seen in Figure 3.4, a large number of new peptides (Figure 3.4 B) and expected glycans (Figure 3.4 C) were seen in the mass spectra. Observation of a single high peak for a complex biantennary glycan as the dominant glycoform in the sample was consistent with previous

reports[23,24]. Appearance of a significant number of new peptides, upon treatment of the sample with PNGase F, provided further proof that our original "glycopeptide" fraction did contain mostly glycopeptides.

3.5.2 Enrichment using in-house made cellulose columns

The rationale for using non-commercial cellulose columns for the next set of experiments was to have the ability to customize parameters such as column packing volumes and flow-rates, which affect sample load capacity and separation efficiency. Another important factor that needed to be improved upon was the sample introduction procedure. Ideally, a sample is introduced into a column as a very narrow zone in order to achieve the best possible separation efficiency. Our idea was to introduce a thin layer of C_{18} material at the top of the column to trap all peptides in an aqueous sample. Once the peptides bound to this material, it may be washed with water for desalting and to remove any detergents used in prior sample preparation steps. When the column is later washed with a "peptide eluting" solution, both peptides and glycopeptides are desorbed from the hydrophobic C_{18} and enter the cellulose phase, where glycopeptides are likely to remain bound and peptides to be washed off. We found that columns designed according to specifications listed in the Experimental section worked well for the intended purpose.

To investigate the extraction efficiency relative to non-retained glycopeptides, i.e. how much glycopeptide material remained in the "peptide" fraction, an additional extraction of the "peptide" fraction was performed and relative peak intensities in the mass spectra of the two "glycopeptide" fractions were compared, as shown in Figure 3.5. It is clear that not all glycopeptide material was recovered in the first extraction as

glycopeptides were observed in the second extraction sample, albeit at a significantly lower level. However, no glycopeptides were detected in the "peptide" fraction after a second extraction. This suggests that, depending on the initial complexity and concentration of a sample with respect to the total peptide/glycopeptide content, it may be necessary to carry out a few successive extractions to ensure a maximum recovery of glycopeptides.

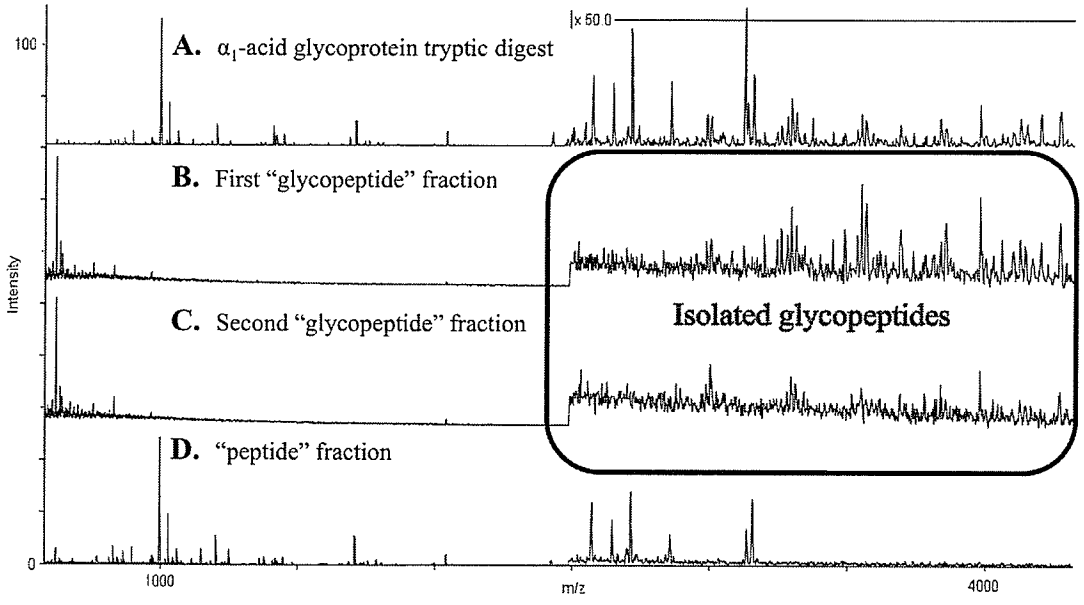

Figure 3.5. Reflector mode mass spectra of whole untreated tryptic digest of bovine α_1-acid glycoprotein (A). First "glycopeptide" fraction (B), second "glycopeptide" fraction (C), and "peptide" fraction (D) of the tryptic digest as separated on a cellulose column. Spectra were acquired on a Manitoba/Sciex MALDI QqTOF instrument in the positive ion mode.

3.5.3 LTQ-OrbiTrap™ experiments

The next set of experiments were carried out with the intention of trying to identify all glycopeptides present in the "glycopeptide" fractions, as well as to examine the efficiency of our extraction method further with the help of a more sensitive instrument. This would ideally be done on an LC-MS system, primarily because (i) it would allow detection of very large glycopeptides in their multiply charged states, which is not possible by MALDI MS, and (ii) separation of the mixture components by LC prior to ionization would eliminate any competitive ionization-suppression effects, as observed in MALDI or direct infusion ESI sample profiling experiments[26,27]. The Thermo LTQ-OrbiTrap™ instrument with a front-end LC was chosen for this purpose, as it is currently one of the most sensitive instruments on the market and because its OrbiTrap mass analyzer offers exceptional mass accuracy and resolution[10,28].

Fractions of the α_1-acid glycoprotein mixture sample were analyzed again using this setup according to the method described in the Experimental section. Since ESI leads to many multiply charged states, raw ESI mass spectra had to be deconvoluted in order to obtain "pseudo-MALDI" mass spectra, where each species is identified by a single peak corresponding to $[M+H]^+$. Deconvoluted mass spectra are presented in Figure 3.6. Direct comparison may be drawn between mass spectra of Figure 3.5 B&C and those of Figure 3.6 B&C, as well as between spectra of Figure 3.5 C and Figure 3.6 A. We were able to identify a large number of glycopeptides in the "glycopeptide" fractions on the basis of amino acid sequences of expected glycopeptides, as well as glycan-specific CID product ions at 204 and 366 *m/z*, which correspond to GlcNAc and HexGlcNAc fragments, respectively. However, as both glycoproteins in the sample contain cysteines and because

the sample was not reduced and alkylated, there were additional glycopeptides present with disulfide linkages, which are not reported here. Also, comparing the mass spectra of "glycopeptide" fractions (Figures 3.5 B and 3.6 B) and mass spectra of deglycosylated "glycopeptide" fractions (Figure 3.6 D), it is evident from the large number of new peptides generated upon deglycosylation that other, non-predicted glycopeptides were also present in our "glycopeptide" fractions. A list of glycopeptides from human α_1-acid glycoprotein related to expected amino acid sequences is presented in Table 3.1. A comparable number of glycopeptides from the human glycoprotein variant were also identified (not shown).

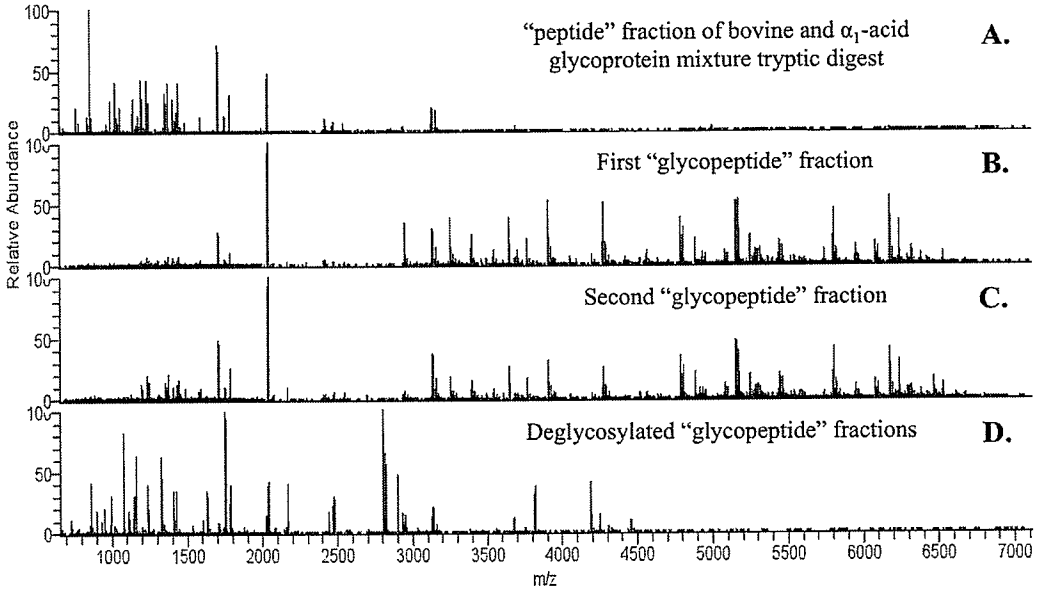

Figure 3.6. Deconvoluted mass spectra of tryptic digest of a bovine and human α_1-acid glycoprotein sample mixture "peptide" fraction (A), first "glycopeptide" fraction (B), and second "glycopeptide" fraction (C). The deglycosylated "glycopeptide" fraction sample was obtained by treatment of combined "glycopeptide" fractions with PNGase F. Mass spectra were acquired on a Thermo LTQ OrbiTrap™ instrument in the positive ion mode.

Table 3.1. Human α_1-acid glycoprotein glycopeptides observed in "glycopeptide" fraction of the tryptic digest of bovine and human α_1-acid glycoprotein mixture.

Peptide sequence	Glycan composition	*m/z* Theoretical	*m/z* Observed	Mass accuracy (ppm)
LVPVPITNATLDQITGK	$Hex_5HexNAc_4Neu5Ac_2$	3984.7938	3984.7880	1.4444
	$Hex_6HexNAc_5Neu5Ac_1$	4058.8306	4058.8265	1.0091
	$FucHex_6HexNAc_5Neu5Ac_1$	4204.8885	4204.8994	-2.5860
	$Hex_6HexNAc_5Neu5Ac_2$	4349.9260	4349.9355	-2.1963
	$Fuc_1Hex_6HexNAc_5Neu5Ac_2$	4495.9839	4495.9944	-2.3429
	$Hex_6HexNAc_5Neu5Ac_3$	4641.0214	4641.0303	-1.9206
	$Fuc_1Hex_6HexNAc_5Neu5Ac_3$	4787.0793	4787.0808	-0.3079
SVQEIQATFFYFTPNK	$Hex_6HexNAc_5Neu5Ac_1$	4198.7629	4198.7739	-2.6022
	$Hex_6HexNAc_5Neu5Ac_2$	4489.8583	4489.8709	-2.7876
	$Hex_6HexNAc_5Neu5Ac_3$	4780.9537	4780.9631	-1.9632
	$FucHex_6HexNAc_5Neu5Ac_3$	4927.0116	4927.0195	-1.5985
	$Hex_7HexNAc_6Neu5Ac_1$	4563.8951	4563.9109	-3.4545
	$Hex_7HexNAc_6Neu5Ac_2$	4854.9905	4855.0004	-2.0342
	$Fuc_1Hex_7HexNAc_6Neu5Ac_2$	5001.0484	5001.0581	-1.9228
	$Hex_7HexNAc_6Neu5Ac_3$	5146.0859	5146.1033	-3.3688
	$Fuc_1Hex_7HexNAc_6Neu5Ac_3$	5292.1459	5292.1438	0.3866
	$Fuc_1Hex_7HexNAc_6Neu5Ac_4$	5583.2392	5583.2612	-3.9325
NEEYNK	Hex5HexNAc4	2418.9288	2418.9350	-2.5805
	Hex5HexNAc4Neu5Ac1	2710.0242	2710.0248	-0.2184
	Hex5HexNAc4Neu5Ac2	3001.1196	3001.1165	1.0156
	FucHex5HexNAc4Neu5Ac1	2856.0821	2856.0718	3.5811
	FucHex5HexNAc4Neu5Ac2	3147.1775	3147.1706	2.1823
	Hex6HexNAc5	2784.0610	2784.0598	0.4088
	Hex6HexNAc5Neu5Ac1	3075.1564	3075.1532	1.0139
	Hex6HexNAc5Neu5Ac2	3366.2518	3366.2501	0.4985
	Hex6HexNAc5Neu5Ac3	3657.3472	3657.3487	-0.4271
	FucHex6HexNAc5Neu5Ac2	3512.3097	3512.3088	0.2528
	FucHex6HexNAc5Neu5Ac3	3803.4051	3803.4015	0.9328
ENGTISR	$Hex_6HexNAc_5Neu5Ac_1$	3055.1989	3055.2014	-0.8186
	$Hex_6HexNAc_5Neu5Ac_2$	3346.2943	3346.2922	0.6332
	$Hex_6HexNAc_5Neu5Ac_3$	3637.3897	3637.3861	0.9922
	$Fuc_1Hex_6HexNAc_5Neu5Ac_1$	3201.2568	3201.2617	-1.5216
	$Fuc_1Hex_6HexNAc_5Neu5Ac_2$	3492.3522	3492.3537	-0.4412
	$Fuc_1Hex_6HexNAc_5Neu5Ac_3$	3783.4476	3783.4427	1.2869
	$Hex_7HexNAc_6Neu5Ac_1$	3420.3311	3420.3398	-2.5527
	$Hex_7HexNAc_6Neu5Ac_2$	3711.4265	3711.4299	-0.9191
	$Hex_7HexNAc_6Neu5Ac_3$	4002.5219	4002.5168	1.2665
	$Hex_7HexNAc_6Neu5Ac_4$	4293.6173	4293.6255	-1.9031
	$Fuc_1Hex_7HexNAc_6Neu5Ac_1$	3566.3890	3566.3885	0.1399
	$Fuc_1Hex_7HexNAc_6Neu5Ac_2$	3857.4844	3857.4889	-1.1694
	$Fuc_1Hex_7HexNAc_6Neu5Ac_3$	4148.5798	4148.5735	1.5063

We were also interested in comparing ionization efficiencies of sialylated and desialylated glycopeptides. As seen in Figure 3.7 A and B, there is no significant difference in signal intensity between the two types relative to a common peptide peak. This was not the case in MALDI experiments (Figure 3.3), where signal due to sialylated species was significantly lower. This shows one advantage of LC-ESI method over MALDI, as described earlier. Nearly complete disappearance of all peaks observed in the "glycopeptide" fraction (Figure 3.7 A) and appearance of completely new peaks in the desialylated "glycopeptide" fraction, together with a general shift in peak distribution towards lower *m/z*, which is consistent with loss of sialic acid, are indicative of a high extent in sialylation occurring in sample glycoproteins, as expected.

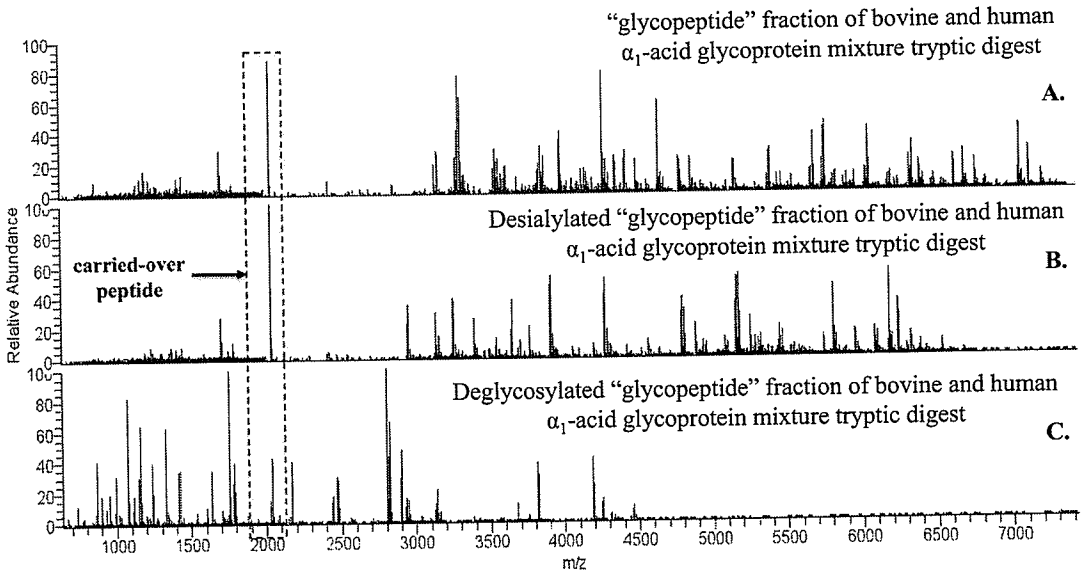

Figure 3.7. Deconvoluted ESI mass spectra of tryptic digests of bovine and human α_1-acid glycoprotein sample mixture made of combined "glycopeptide" fractions (A), desialylated "glycopeptide" fraction (B), and deglycosylated "glycopeptide" fraction (C). Mass spectra were acquired on a Thermo LTQ OrbiTrap^{TM} instrument in the positive ion mode.

Bovine fetuin, a well-characterized glycoprotein containing both *N*- and *O*-linked sites of glycosylation[29], was the subject of further experiments. All predicted glycopeptides were observed in the "glycopeptide" fraction of this sample with very low amount of non-glycopeptide carry-over (Figure 3.8). Glycopeptides observed are listed in Table 3.2. It is important to point out that even a relatively large *O*-glycosylated peptide with amino acid sequence VTCTLFQTQPVIPQPQPDGAEAEAPSAVPDAAGPTPSAA GPPVASVVVGPSVVAVPLPLHR (~5958 Da) was retained by the column. This demonstrated that glycopeptides with both short and very long peptide moieties could be effectively extracted by our method.

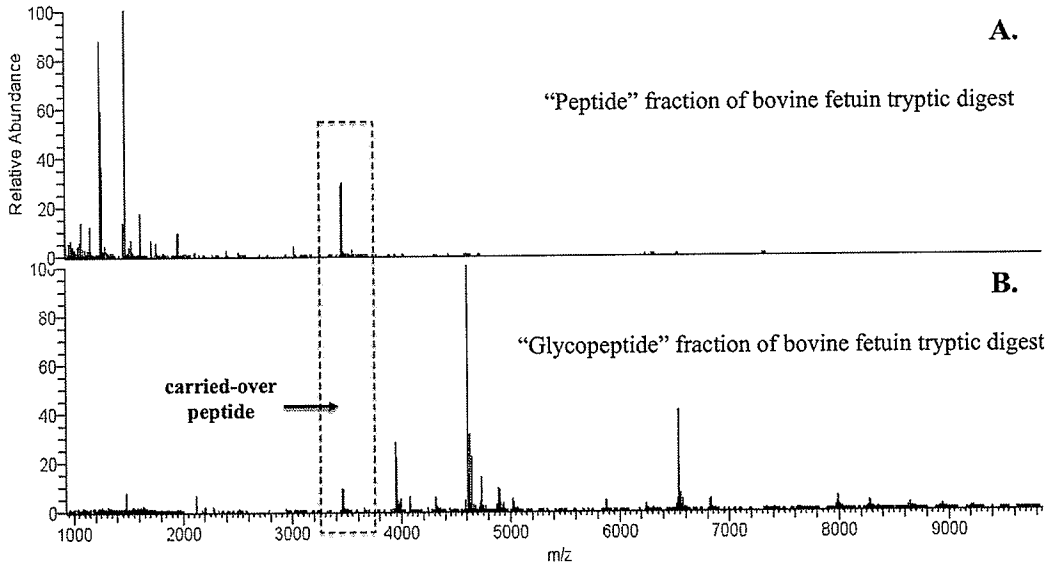

Figure 3.8. Deconvoluted ESI mass spectra of tryptic digest of bovine fetuin "peptide" (A) and glycopeptides (B) fractions. Mass spectra were acquired on a Thermo LTQ OrbiTrap™ instrument in the positive ion mode.

Table 3.2. Glycopeptides observed in "glycopeptide" fraction of fetuin tryptic digest sample.

Peptide sequence	Glycan composition	*m/z* Theoretical	*m/z* Observed	Mass accuracy (ppm)
L*CPD*CPLLAPLNDSR	$Hex_5HexNAc_4Neu5Ac_2$	3945.6131	3945.6208	-1.9337
	$Hex_5HexNAc_4Neu5Ac_3$	4236.7085	4236.7148	-1.4740
	$Hex_5HexNAc_5Neu5Ac_2$	4148.6925	4148.6938	-0.3169
	$Hex_6HexNAc_5Neu5Ac_2$	4310.7453	4310.7475	-0.4975
	$Hex_6HexNAc_5Neu5Ac_3$	4601.8408	4601.8472	-1.3842
	$Hex_6HexNAc_5Neu5Ac_4$	4892.9362	4892.9410	-0.9830
KL*CPD*CPLLAPLNDSR	$Hex_5HexNAc_4Neu5Ac_2$	3945.6131	3945.62082	-1.9337
	$Hex_5HexNAc_4Neu5Ac_3$	4236.7085	4236.71475	-1.4740
	$Hex_5HexNAc_5Neu5Ac_2$	4148.6925	4148.69382	-0.3169
	$Hex_6HexNAc_5Neu5Ac_2$	4310.7453	4310.74745	-0.4975
	$Hex_6HexNAc_5Neu5Ac_3$	4601.8408	4601.84717	-1.3842
	$Hex_6HexNAc_5Neu5Ac_4$	4892.9362	4892.94103	-0.9830
VT*CTLFQTQPVIPQPQPDG AEAEAPSAVPDAAGPTPSA AGPPVASVVVGPSVVAVPL PLHR[a]	$Hex_2HexNAc_2Neu5Ac_2$	7327.5874	7327.6074	-2.7242
	$Hex_2HexNAc_2Neu5Ac_3$	7618.6828	7618.6926	-1.2773
	$Hex_3HexNAc_3Neu5Ac_3$	7983.8150	7983.8211	-0.7567
	$Hex_3HexNAc_3Neu5Ac_4$	8274.9104	8274.9253	-1.7899
	$Hex_3HexNAc_3Neu5Ac_5$	8566.0058	8566.0252	-2.2638
	$Hex_4HexNAc_4Neu5Ac_4$	8640.0426	8640.0575	-1.7212
	$Hex_4HexNAc_4Neu5Ac_5$	8931.1380	8931.1298	0.9223
	$Fuc_1Hex_3HexNAc_4Neu5Ac_5$	8915.1431	8915.1445	-0.1505
RPTGEVYDIEIDTLETT*CH VLDPTPLAN*CSVR	$Hex_5HexNAc_4Neu5Ac_2$	5876.5404	5876.5317	1.4839
	$Hex_6HexNAc_5Neu5Ac_2$	6241.6725	6241.6688	0.5954
	$Hex_6HexNAc_5Neu5Ac_3$	6532.7680	6532.7808	-1.9532
	$Hex_6HexNAc_5Neu5Ac_4$	6823.8635	6823.8810	-2.5777
VVHAVEVALATFNAESNGS YLQLVEISR	$Hex_6HexNAc_5Neu5Ac_3$	5877.5738	5877.5879	-2.4057

[a]*O*-linked glycopeptides. Sites of glycosylation were not determined at the time of writing. The asterisks denote carboxymethylation of cysteines obtained during the alkylation step.

To examine performance of the method with more complex sample types, a tryptic digest of a 10-protein sample mixture, as described in the Experimental section, was used. Ovalbumin, transferrin, and α-lactalbumin were the three glycoproteins in this sample. Mass spectra are given in Figure 3.9. Two of the most prominent peaks in the mass spectrum of the "glycopeptide" fraction (Figure 3.9 B) correspond to the two glycopeptides predicted from transferrin with dominant biantennary disialo glycoforms[15]. This is despite the fact that transferrin constituted only ~4% of the total molar protein

content of the sample. A large number of glycopeptides originating from the ovalbumin peptide YNLTSVLMAMGITDVFSSSANLSGISSAESLK were also observed, however due to presence of more than 20 known glycoforms[30], all glycopeptide ion peaks related to ovalbumin were of relatively low abundance. We were unable to identify any glycopeptides derived from α-lactalbum and are currently investigating this further. Additionally, it is apparent from the number of observed peptide peaks in the "glycopeptide" fraction that carry-over rate of non-specific peptides rises substantially with increased sample complexity, especially when glycoproteins represent only a minor fraction of the total sample material.

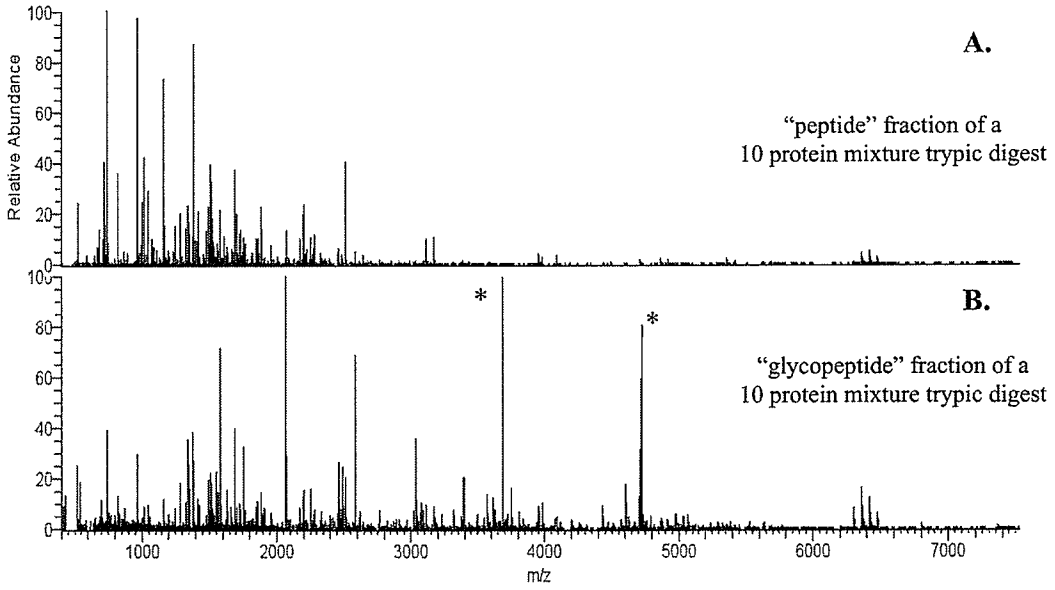

Figure 3.9. Deconvoluted ESI LTQ OrbiTrap™ mass spectra of tryptic digest of a 10-protein sample mixture "peptide" fraction (A) and "glycopeptide" fraction (B). Mass spectra were acquired on a Thermo LTQ OrbiTrap instrument in a positive ion mode. Peaks marked by the asterisks are abundant disyalylated glycopeptides from bovine transferrin (CGLVPVLAENYNK and QQQHLFGSNVTDCSGNFCLFR, respectively) with carboxymethylated cysteine residues.

3.5.4 Future work

While it is accepted that the main mode of interaction of glycopeptides with cellulose is through the hydrophilic oligosaccharide moieties, non-specific retention of peptides takes place and it is not well explained why. A number of factors need to be considered and tested here in order to formulate a good model. If the net hydrophilicity of a molecule can be related to the number of functional groups capable of hydrogen bonding, as well as to the absence of those that are not, one may speculate that any peptide, even if composed entirely of "hydrophilic" amino acids, will have a much lower hydrophilic character than any glycan found on glycopeptides, and would thus be expected to have a significantly lower retention. It is important to remember that peptides do interact within themselves mainly through ionic and hydrophobic interactions. Even as ionic interactions are cancelled by the use of an ion-pairing agent such as TFA, peptides in a sample may still interact with each other. Likewise, glycopeptides may also aggregate via hydrophilic glycan moieties. Thus, both of these types of hydrophobic and hydrophilic interactions may be responsible for the incomplete initial recovery of glycopeptides and non-specific carry-over of peptides, as they affect the net interactions between sample and stationary phase. This is something that certainly requires further investigation and improvement.

3.6 Conclusions

A simple and efficient method for on-column isolation of glycopeptides from complex proteolytic digests using cellulose as solid-phase extraction material was developed. Glycopeptides were enriched in good yields for further

glycoproteomic/glycomic studies. This technique offers an advantage over previously reported in-solution methods in its potential for on-line enrichment with subsequent LC-MS analysis experiments. The Thermo LTQ-OrbiTrap^{TM} instrument proved to be a powerful tool for detection and identification of glycopeptides in complex mixtures, providing exceptional sensitivity, resolution, and mass accuracy even at high *m/z* values, which is not typically possible with most current MALDI-based instruments. Although this study demonstrated utility of cellulose for on-column isolation of glycopeptides, more experiments to probe the extraction efficiency of cellulose material need to be performed in order to establish this method as a norm for general glycopeptide enrichment in glycoproteomic/glycomic studies. Factors such as size of cellulose particles, physical design of the column and construction materials involved, extent of compression of bulk cellulose packing material in the column, solvents used, as well as many others, are expected to exert some influence on separation efficiency and would clearly need to be tested and optimized in the course of future work.

3.7 Acknowledgments

The authors thank members of the Standing/Ens lab for help with the use of their mass spectrometer, as well as members of the Khoo lab (Biological Chemistry Institute, Academia Sinica, Taiwan) for helpful discussions. The Natural Sciences and Engineering Council of Canada (NSERC), the Canada Research Chairs program (CRC), Canadian Foundation for Innovation (CFI), and the National Science Council (Taiwan) are acknowledged for funding.

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Chapter 4:

Use of aniline as an additive in MALDI matrices for on-target derivatization and analysis of oligosaccharides by MALDI MS.

4.1 Authors Contributions

All experiments pertaining to this work were designed and conducted by Sergei I. Snovida. All figures and manuscript drafts were also created by Sergei I. Snovida. Dr. V. C. Chen provided help with acquisition of MS/MS data and revisions of the manuscript. Dr. H. Perreault was responsible for editing the final version of the manuscript prior to initial submission to the journal of Analytical Chemistry. All authors contributed equally to final revision of the manuscript prior to its publication.

Use of a Novel DHB/Aniline MALDI Matrix for Improved Detection and On-Target Derivatization of Glycans.

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Published: *Anal. Chem.* **2006**, *78* (24), 8561-8568

4.2 Abstract

N-linked glycans derived from human and bovine α_1-acid glycoprotein, as well as chicken egg white albumin, were analyzed by MALDI-TOF mass spectrometry using a novel MALDI matrix consisting of 2,5-dihydroxybenzoic acid (DHB) and aniline. A significant increase in signal was observed for these oligosaccharides relative to the signal obtained when unmodified DHB was used as a matrix for the same set of samples. The use of aniline/DHB matrix also led to facile on-target derivatization of the glycans via non-reductive amination, as aniline was found to form a stable Schiff base with the reducing end GlcNAc residue without the need for prolonged incubation periods and elevated temperatures. Both native and derivatized glycans ionized as sodium adducts and had similar MS/MS fragmentation patters consisting mainly of Y/B-cleavage ions. In our experiments we obtained evidence for persistence of the derivatization reaction in the solid phase; i.e. the reaction appeared to be taking place even after the sample-matrix spot had dried. This is the first report of such solid-phase on-target derivatization of carbohydrates for subsequent analysis by MALDI mass spectrometry.

4.3 Introduction

Glycobiology has experienced a rapid growth as a field in science due to recognition of many key biological processes in which carbohydrates play a key role[1-4]. Cell adhesion, protein folding, immune response, signaling and trafficking of biomolecules are only some of the important events where glycans are implicated as central participants. Modification of proteins by glycosylation has been a popular research topic for a number of years now[5-7]. Today, it is a widely accepted fact that this type of modification affects the function, localization, and lifetime of many proteins synthesized by a cell. It is therefore important to have efficient and reliable tools for characterization of oligosaccharides in order to make further advances in the field.

Much of the success in the field of glycobiology is related to the development of mass spectrometry-based methods[8,9]. Introduction of electrospray ionization (ESI)[10] and matrix assisted laser desorption ionization (MALDI)[11] has revolutionized analytical science: the techniques have made possible the ionization and detection of very low amounts of large biomolecules without the need for extensive modification of analytes. One of the main advantages of using MALDI relative to ESI is the use of static samples, which can be redissolved and treated for subsequent analysis. This is crucial when one is working with limited quantities of biological material. Moreover, MALDI results in predominantly singly charged ions, which is advantageous for quick interpretation of a given mass spectrum.

Analysis of oligosaccharides by mass spectrometry (MS) has been a challenge for many years due in part to the limited capacity of these compounds to ionize[8]. Although positive ionization is enhanced when amino or *N*-acetyl groups are present, most charged

carbohydrates are acidic and are better analyzed in the negative ionization mode. To circumvent this problem, glycans may be chemically modified to improve their ionization efficiency. Several derivatization procedures have been developed and are successfully used today in routine analyses[12,13]. A large portion of these reactions is based on reductive amination with a suitable amine reagent via formation of a Schiff base, according to Scheme 1. Although the use of chromophore/fluorophore amines for derivatization of glycans was originally developed to aid detection of sugars by UV/fluorescence detectors following separation of mixtures by liquid chromatography (LC) or electrophoresis-based techniques, some of these reagents have also been found to work well for the purpose of MALDI.

To reduce the possibility of sample contamination or loss, there is a general drive towards minimizing the number of handling steps in sample preparation procedures. As a result, a number of recently reported methods employ non-reductive amination reactions[14,15], which simplify post-derivatization clean-up and the overall derivatization process while still leading to improved analysis of oligosaccharides by MALDI mass spectrometry. These methods are based on formation of a stable Schiff base/glycosylamine with a nearly complete conversion of the native glycans. In this article, we report on how such protocols may be optimized further by incorporating an amine reagent into a common MALDI matrix, 2,5-dihydroxybenzoic acid (DHB), and performing on-target derivatization of glycans. We used aniline as this reagent and describe for the first time several unique features specifically pertaining to on-target nonreductive amination of carbohydrates.

4.4 Experimental

4.4.1 Materials and reagents

Milli-Q water was used in all preparations. HPLC-grade acetonitrile (ACN) was purchased from Fischer Scientific (Fair Lawn, NJ, USA). Ovalbumin (chicken egg white albumin), human and bovine α_1-acid glycoprotein, 2,5-dihydrohybenzoic acid (DHB), and aniline were obtained from Sigma (St. Louis, MO, USA). PNGase F endoglycosidase enzyme was purchased from ProZyme (San Leandro, CA, USA).

4.4.2 Sample preparation

One milligram of a protein was dissolved in 200 μL of 25 mM ammonium bicarbonate buffer at pH 7.8 in a 0.6 mL microcentrifuge tube. PNGase enzyme solution (5 μL, as provided by the manufacturer) was added to the tube and the contents were mixed using an agitator for about one minute. The mixture was incubated at 37 °C for 20 hours, with additional mixing after the first 10 hours. Detached glycans were isolated by loading 10 μL of the digestion mixture onto a C_{18} reverse-phase (RP) column, which was prepared by a previously described procedure[16], and flushing it with 200 μL of water to elute the sugars. The effluent was collected in a 0.6 mL tube and its volume was reduced to 50 μL on a Speed Vac system.

Asialo glycans from human and bovine α_1-acid glycoprotein were obtained by initially adding 10 μL of the PNGase F digest mixture to 40 μL of 1% trifluoroacetic acid (TFA) solution in a 0.6 mL tube and incubating the solution at 75 °C for 30 minutes to remove sialic acids. Upon completion of this step, material was lyophilized to remove

TFA and resuspended in 50 μL of water. Asialo glycans were then isolated from the mixture using RP-LC as described above.

A fraction of a tryptic digest of bovine α_1-acid glycoprotein containing glycoforms of npeyNk tryptic glycoconjugate and another unmodified tryptic peptide of 810.37 Da was obtained by a LC method described previously[16]. Material was lyophilized and re-dissolved in 10 μL of water. PNGase F solution (2 μL) was added to a 5 μL portion of the solution in a 0.6 mL tube and the mixture was diluted to 50 μL with 25 mM ammonium bicarbonate buffer (pH 7.8). The remaining 5 μL of the solution was also diluted to 50 with the 25 mM ammonium bicarbonate. Both solutions were incubated at 37 °C for 5 hours upon which they were lyophilized to remove ammonium acetate salt and material in each tube was re-dissolved in 50 μL of water.

4.4.3 MALDI matrix preparation

DHB matrix solution was prepared by dissolving 100 mg of DHB in 1 mL of a 1:1 solution of water and ACN. The DHB/aniline matrix solution was prepared by adding 20 μL of neat aniline to the DHB matrix solution. Samples were deposited onto a polished steel MALDI target by mixing 0.75 μL of sample and matrix solutions on-target and allowing the spot to dry by evaporation.

4.4.4 Mass spectrometric measurements

All single mass spectra were acquired on a Bruker Biflex IV MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) in the positive ion reflecting mode. MS/MS experiments were performed on the University of Manitoba/Sciex MALDI-

QqTOF instrument (Department of Physics, University of Manitoba, Canada; PE Sciex, Mississauga, ON, Canada).

4.4.5 Online tools

Monosaccharide compositions of glycans were calculated using GlycoMod tool from ExPASy Proteomics tools[17].

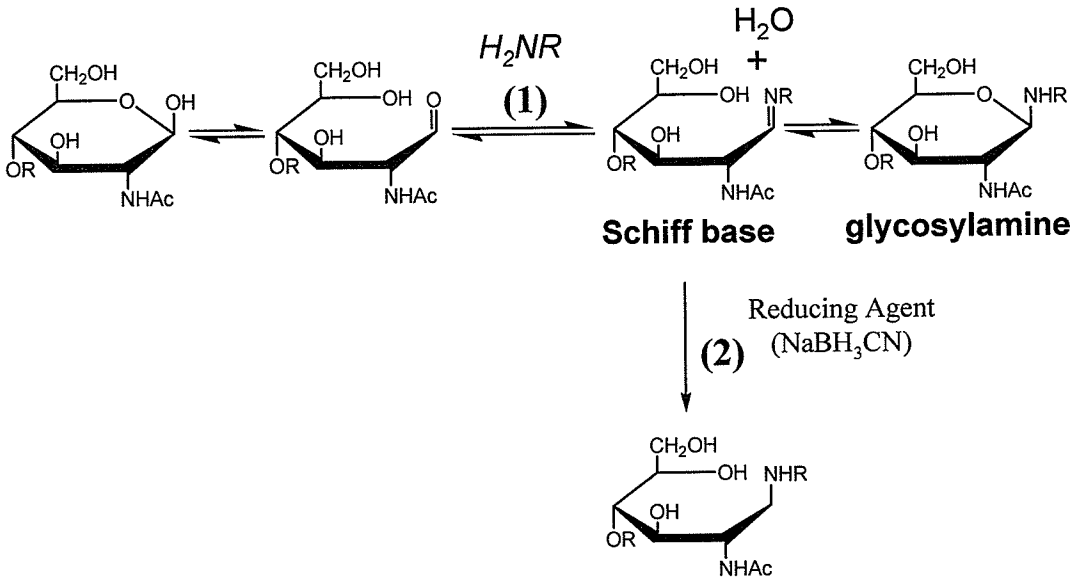
4.5 Results and discussion

Our initial goal in the study was to develop a procedure for non-reductive amination of carbohydrates employing aniline as an amine reagent owing to its low cost and availability. We chose to use glycans from bovine α_1-acid glycoprotein released using PNGase glycosidase as model sugars for this purpose, as glycosylation in this glycoprotein has been extensively described previously in the literature[18]. Most importantly, these glycans occur mainly as sialylated biantennary structures and sialic acids may be easily removed without affecting the rest of the glycan[19]. Thus, bovine α_1-acid glycoprotein served as a source of relatively simple mixtures of both sialylated and non-sialylated glycans *N*-linked glycans.

4.5.1 Preliminary results

Adopting our experiments to follow a protocol developed by Lattova *et. al.*[20] involving a solvent extraction step to remove excess reagent, aniline in our case, we were successful in achieving our intent with relative ease (data not shown). However, complete conversion of the glycans into their derivative form was never observed: native forms of

the glycans still represented a significant portion of the post-derivatization sample, based on the relative signal intensities in the MALDI-TOF mass spectra. Changes in pH of the reaction medium, reaction temperatures and time did not produce a noteworthy effect, and the reaction appeared to be roughly only 50% efficient in terms of labeled/unlabeled glycan signal intensity, regardless of the conditions used. This was unlike the reaction with phenylhydrazine, where nearly complete derivatization was observed in terms of mass spectral detection[20].



Scheme 4.1. Labeling of Reducing Sugars by Reductive Amination.

(1) a reactive primary amine is added to the mixture producing a Schiff Base intermediate; (2) reduction of the Schiff Base by a mild reducing agent irreversibly yields a labeled sugar.

4.5.2 On-target reactions

We recognized that formation of Schiff bases is an equilibrium phenomenon (Scheme 4.1), which depends on the concentrations of water and amine reagent. Given that the reaction takes place in an aqueous medium, the Schiff base formation is disfavoured unless a particularly reactive amine is used in relatively high concentration. Once derivatization has taken place, the excess reagent is removed by solvent or cartridge[20,21], leaving labeled oligosaccharides in the aqueous phase. The fact that they do not return to the unlabeled form implies that either the reactive nitrogen in phenylhydrazine is a particularly good nucleophile and leads to formation of an extremely stable Schiff base/glycosylamine or that the reagent is still present in the sample as an impurity after cleanup, thus promoting formation of derivatives. If the latter is the case, the reaction should proceed at the time of drying of the sample spot on a MALDI target, since water is being driven off by evaporation and the apparent reagent concentration increases, which should push the equilibrium towards formation of the desired product. If this is true, it follows that a small amount of the reagent may be introduced at the time of mixing of the sample with a matrix on-target to derivatize the glycans without the need for incubation and elevated temperatures. We decided to explore this possibility further using aniline, as the amount of information in the literature on the use of this label is still fairly limited.

Because of the invariance of efficiency of the aniline reaction in solution with respect to reaction parameters, especially reaction time, aniline did not appear as a particularly good nucleophile and the equilibrium between native and Schiff base glycan forms seemed to establish relatively quickly. To see if we could use the new on-target

approach to reproduce the results from our preliminary work, the matrix was spiked with aniline, and solutions containing native sialo- and asialoglycans glycans from bovine α_1-acid glycoprotein were mixed on-target with the DHB/aniline matrix solution. The amount of aniline added to DHB was optimized on the basis of the minimum amount needed to produce uniform dried crystals throughout a spot. This was found to be about 2% aniline by volume. Equivalent amounts of the samples mixed with DHB-only solution were used as controls. Spots were allowed to dry (approximately 10 minutes), and samples ware analyzed by MALDI-TOF MS. Figure 4.1 compares mass spectra of the glycan samples crystallized in DHB and in DHB/aniline matrices.

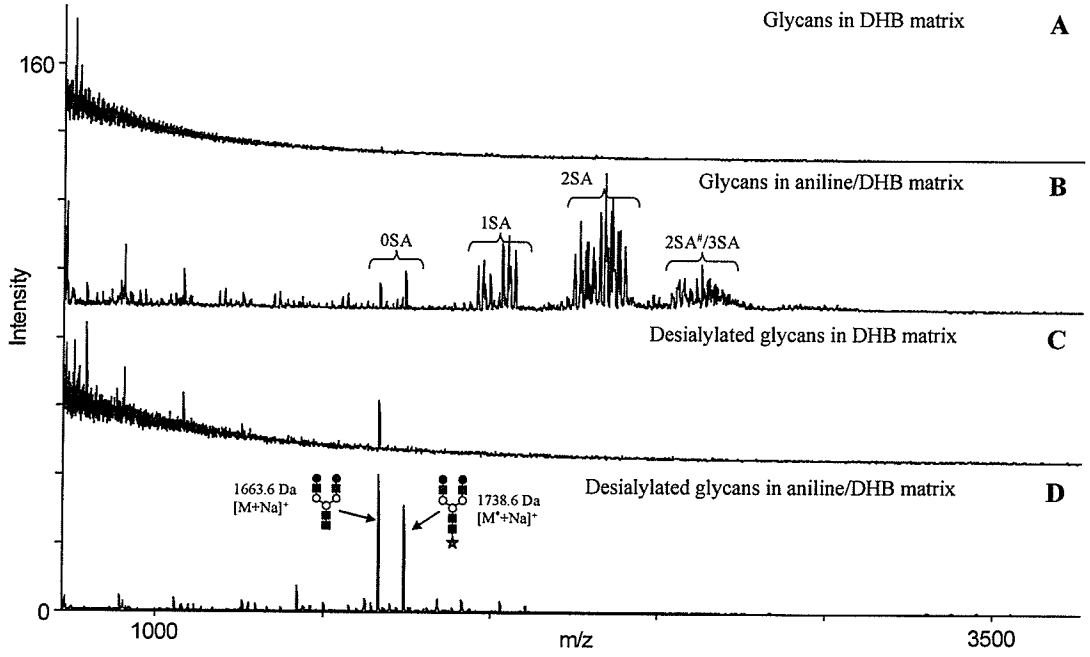


Figure 4.1. MALDI-TOF mass spectra of glycans released from bovine α_1-acid glycoprotein. Peaks are grouped according to the number of sialic acid residues. $2SA^{\#}/3SA$ group contains both disialylated tri-antennary and trisialylated bi-antennary glycans. Same amount of sample was used in all preparations. Asterisk denotes presence of aniline label. Refer to Chart 4.1 for symbol meanings.

N-acetylglucosamine (GlcNAc)

Mass: 221.1 Da
Residual Mass: 203.1 Da

Mannose (Man)

Mass: 180.1 Da
Residual Mass: 162.1 Da

Galactose (Gal)

Mass: 180.1 Da
Residual Mass: 162.1 Da

Sialic acid (NeuAc/NeuGc)

Mass: 309.1/325.9 Da
Residual Mass: 291.1/307.1 Da

Fucose (Fuc)

Mass: 164.1 Da
Residual Mass: 146.1 Da

Aniline label (An)

Mass: 93.1 Da
Label Mass: 75.0 Da

Chart 4.1. Symbols and abbreviations of monosaccharides used in structural diagrams of glycans.

Native and derivatized glycans (mass difference of 75.0 Da) are observed in the DHB/aniline matrix (Figure 4.1 B, D) as Na^+ adduct ions. Native glycans in Figure 4.1 C are detected with significantly higher signal-to-noise ratios relative to their controls (Figure 4.1 A). Note that the sialylated glycans ionized well in the DHB/aniline matrix (Figure 4.1. B) and did not show extensive loss of sialic acid, despite the acidic environment of the matrix. This may be explained by the reaction being performed at room temperature for a brief period of time. Although asialo glycans are detected (region labeled 0SA in Figure 4.1. B), these ions likely result from in-source loss of sialic acid.

An unexpected feature in these results is the improved signal intensity of the native glycans in the aniline-modified DHB matrix. The use of DHB/aniline matrix solution resulted in sample spots composed of very fine crystals compared to those of DHB alone and led to a more uniform sample distribution within a spot. This is produced similar

signal intensities of the native and labeled glycans as well as their relative ratios throughout the spot. For the same amount of material deposited with DHB-only matrix, "sweet spots" yielding comparable signal intensities for the native glycans were not found, despite repeated attempts. This strongly suggests that the presence of aniline in the DHB matrix affects not only homogeneity of sample distribution within a spot, as in ionic liquid matrices[22], but also the absorption of laser energy by the matrix, as well as the energy transfer processes in desorption and ionization of oligosaccharides. These may be enhanced by the formation of DHB/aniline salt, anilinium dihydroxybenzoate, which may better absorb and redistribute laser energy compared to DHB alone. It follows that the development of similar hybrid MALDI matrix system analogous to recently introduced ionic liquid matrices[22], rather than development of new derivatization methods, might be a more practical move toward improving the detection of oligosaccharides by MALDI mass spectrometry. All these hypotheses are currently under investigation and will be subject of our future work.

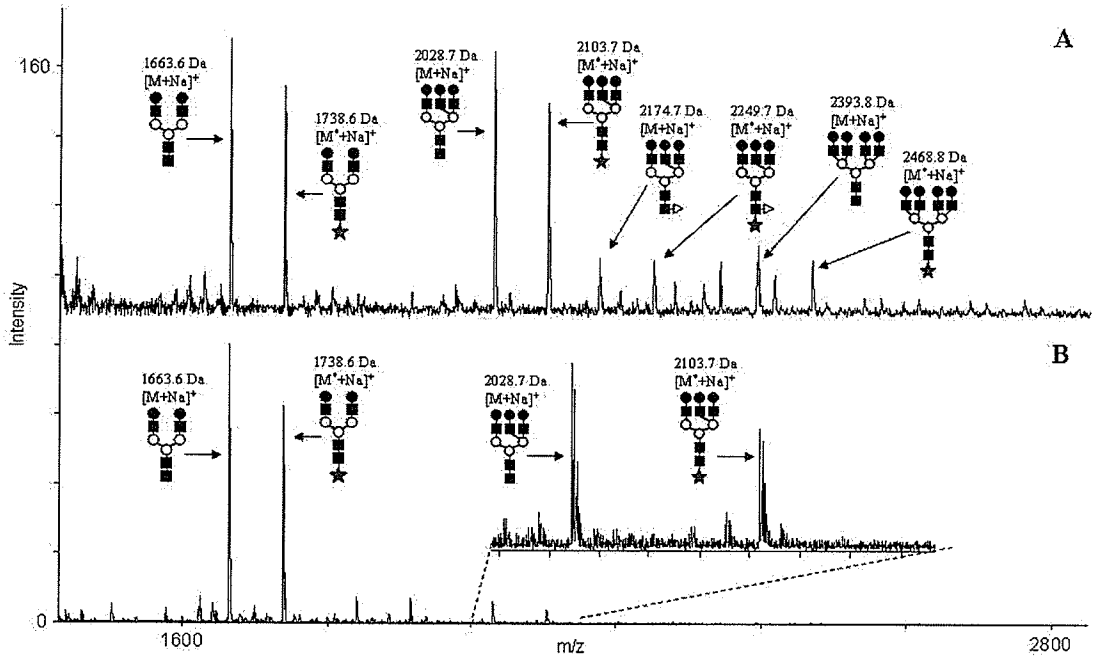


Figure 4.2. MALDI-TOF mass spectra of desialylated glycans released from human α_1-acidglycoprotein (A) and bovine α_1-acid glycoprotein (B) in aniline/DHB matrix. Inset in (B) is the 2000-2150 *m/z* region of the spectrum.

4.5.3 Glycans from human and bovine α_1-acid glycoprotein

Ionic signal suppression is often a major problem in the analyses of complex mixtures[23], particularly proteolytic digests, by MALDI. It arises from the differences in ionization potential of the mixture components. Many compounds may go undetected as a result, which can lead to incomplete characterization of a given sample. In positive ion mode, it is often the case for negatively charged species, such as phosphopeptides and sialylated oligosaccharides. In order to address the issue of signal suppression in our analysis of carbohydrates, the next set of experiments focused on glycans derived from human α_1-acid glycoprotein. These oligosaccharides contain a higher proportion of tri- and tetra-antennary glycans than those from bovine α_1-acid, as well as a significant

proportion of the tri-antennary glycans in fucosylated form[18]. Figure 4.2 shows mass spectra of asialo glycans from both species deposited in DHB/aniline matrix. Native asialo glycans and their aniline derivatives were easily picked out in the spectra. The presence of strong signals for both forms helped identifying the glycans in these mixtures. Moreover, the observed relative abundances of glycans were similar to those reported[18], i.e. suppression effects were minimal among asialo glycans of different antennarities.

Mass spectra of the untreated glycans showed the presence of disialylated biantennary glycans (among other sialylated structures) in a higher *m/z* range, as displayed in Figure 4.3. The number of peaks observed for each sialylated structure of human glycans (Figure 4.3. A) is $n+1$, where n is the number of sialic acid residues. All are singly charged sodium adduct ions in the form of $[M+(x+1)Na\text{-}xH]^+$, where $x=0,1..n$. In contrast, $(n+1)^2$ peaks are seen for each sialylated oligosaccharide structure from bovine α_1-acid glycoprotein (Figure 4.3 B). Bovine glycoprotein contains both NeuAc and NeuGc sialic acids (mass difference of 16 Da) in roughly equal amounts, whereas humans generally lack the enzymatic machinery to synthesize NeuGc, yielding a glycoprotein containing mostly NeuAc sialic acid[24]. Therefore, the ions here may be expressed in the form $[(M+16y)+(x+1)Na\text{-}xH]^+$, where $y=0,1..n$. Data in Figure 4.3 is in agreement with previously published work on the formation of sodium adducts for sialylated glycans[25]. Here again, the use of the DHB/aniline matrix minimized the ionization suppression effects between asialo and sialylated glycans.

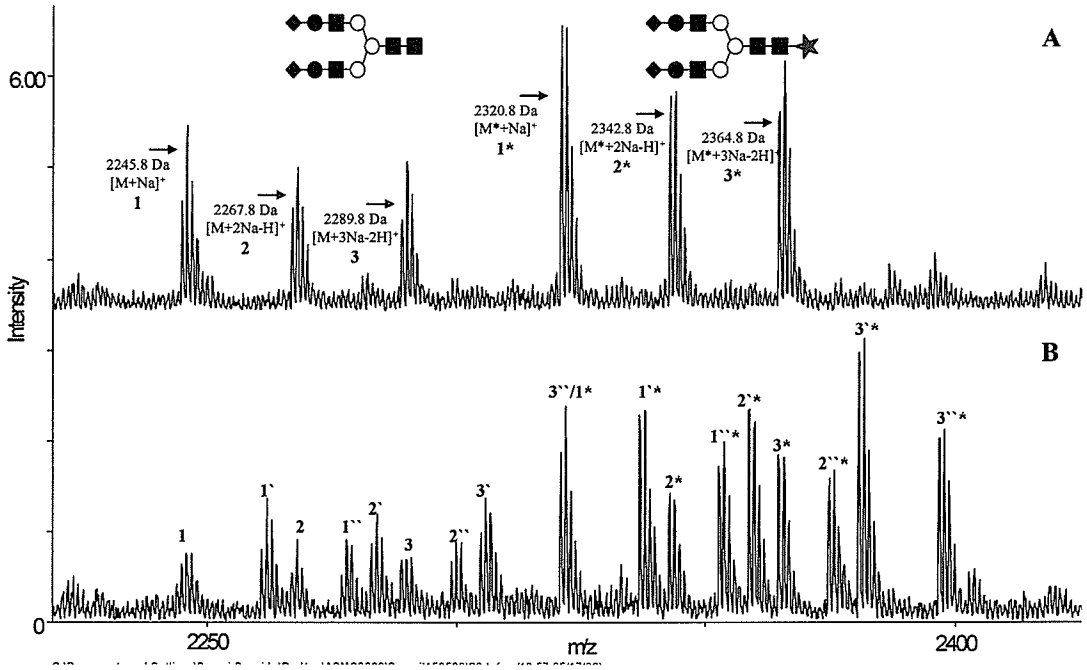
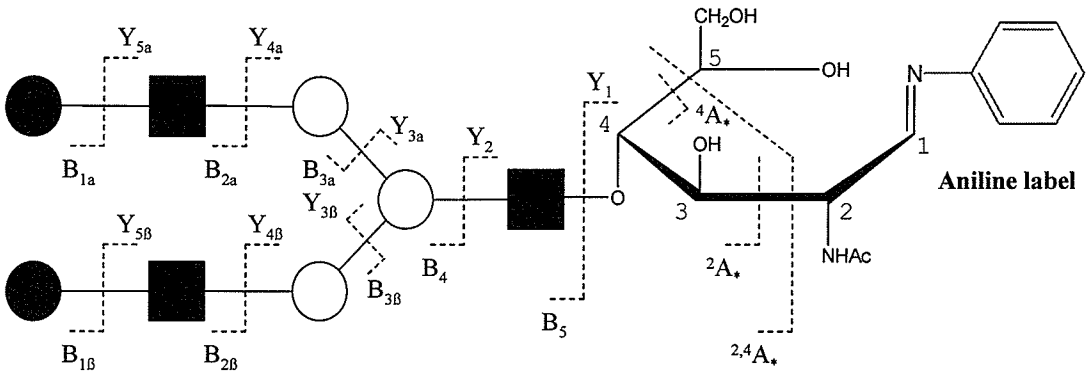


Figure 4.3. Expanded region of the MALDI-TOF mass spectra of native glycans released from human α_1-acid glycoprotein (A) and bovine α_1-acid glycoprotein (B) containing bi-antennary disialylated glycans. The samples were prepared in aniline/DHB matrix. The prime symbol designates a NeuGc replacing a NeuAc sialic acid; asterisks denote derivatives.

4.5.4 Tandem mass spectrometry studies

Collision-induced dissociation spectra of $[M+Na]^+$ ions of native and aniline-derivatized glycans from the same sample were studied next. Tandem mass spectra of bi-antennary glycans of composition $Gal_2GlcNAc_2Man_3GlcNAc_2$ are shown in Figure 4.4. The legend for fragment ions is provided in Scheme 4.2, according to nomenclature proposed by Costello and Domon[26], and some prominent ions detected are listed in Table 4.1.



Scheme 4.2. Observed Fragmentation of $Gal_2GlcNAc_2Man_3GlcNAc_2$ Glycans. MS/MS spectra are given in Figure 4.4; *m/z* values are given in Table 4.1. Ring carbons of the reducing end GlcNAc are labeled 1-5.

The spectra appeared very similar for the most part. Both glycans showed mainly B- and Y-type fragments with comparable relative abundances. Differences were also observed, the most notable being the higher abundance of cross-ring cleavage $^{2}A_{*}$ ions (the asterisk indicates reducing end GlcNAc) of the aniline derivative relative to those of the corresponding native glycan. This could be explained by different equilibria between open and cyclic forms of the reducing end GlcNAc in the native and derivatized glycans. The closed-ring form of GlcNAc is strongly favoured in the case of native structure[27], and for this reason, $^{2}A_{*}$ fragments would be expected to produce small peaks. The high abundance of $^{2}A_{*}$ for the aniline derivative hints that the acyclic form should be more favoured. A strong $^{2,4}A_{*}$ ionic signal and absence of $^{4}A_{*}$ fragments in both spectra suggest that, in the case of an acyclic structure, the C_4-C_5 cleavage occurs after the C_2-C_3 cleavage to yield a $^{2,4}A_{*}$ fragment ion. Thus, abundant simple Y fragments observed in the MS/MS spectrum of the native glycan and abundant $^{2}A/Y$, $^{2,4}A/Y$ ions in the tandem mass spectrum of the aniline derivative suggests that the reducing end of the aniline

derivative is predominantly in the open form and that of the native glycan is in the cyclic form.

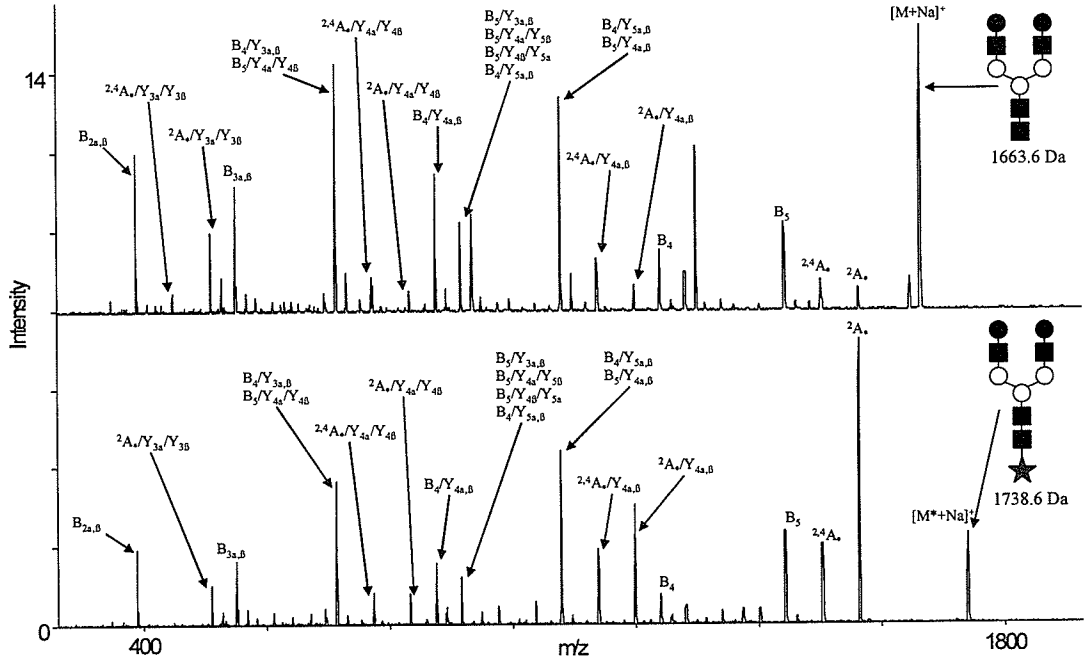


Figure 4.4. MALDI-QqTOF MS/MS spectra of sodiated native (top) and aniline-labeled (bottom) $Gal_2GlcNAc_2Man_3GlcNAc_2$ glycans. All labeled ions are observed as sodium ion adducts; fragments are defined in Scheme 4.2; ion *m/z* values are listed in Table 4.1.

It is important to point out here that the use of DHB/aniline resulted in enhanced quality MS/MS spectra for native glycans. Because more of the sample is ionized per amount deposited on a target, fragment peaks have a much higher signal-to-noise ratio compared to the spectra obtained using DHB alone as a MALDI matrix. The introduction of aniline allows more efficient structural characterization of oligosaccharides by MALDI-TOF MS.

Table 4.1. Intense Ions Observed in the MS/MS Spectra of Sodiated Native and Aniline-labeled $Gal_2GlcNAc_2Man_3GlcNAc_2$ Glycans Presented in Figure 4.4.

Ion	*Ion m/z* (Da)
$B_{2\alpha,\beta}$	388.1
Y_2	447.2[a]
$^{2,4}A_*/Y_{3\alpha}/Y_{3\beta}$	448.2[b]
$^{2}A_*/Y_{3\alpha}/Y_{3\beta}$	509.1
$B_{3\alpha,\beta}$	550.2
$B_4/Y_{3\alpha,\beta}$, $B_5/Y_{4\alpha}/Y_{4\beta}$	712.2
$Y_{3\alpha,\beta}/Y_{4\alpha,\beta}$	771.3
$^{2,4}A_*/Y_{4\alpha}/Y_{4\beta}$	772.3
$^{2}A_*/Y_{4\alpha}/Y_{4\beta}$	832.3
$B_4/Y_{4\alpha,\beta}$	874.3
$B_5/Y_{3\alpha,\beta}$, $B_5/Y_{4\alpha}/Y_{5\beta}$, $B_5/Y_{4\beta}/Y_{5\alpha}$, $B_4/Y_{5\alpha,\beta}$	915.3
$B_4/Y_{5\alpha,\beta}$, $B_5/Y_{4\alpha,\beta}$	1077.4
$Y_{4\alpha,\beta}/Y_{5\alpha,\beta}$	1136.4
$^{2,4}A_*/Y_{4\alpha,\beta}$	1137.4
$^{2}A_*/Y_{4\alpha,\beta}$	1197.4
B_4	1239.4
B_5	1442.5
$Y_{5\alpha,\beta}$	1501.6
$^{2,4}A_*$	1502.6
$^{2}A_*$	1562.6

[a]Ions in MS/MS spectrum of the native glycans. [b]Ions in MS/MS spectrum of aniline-labeled glycan.

4.5.5 Simultaneous analysis of peptides, glycopeptides, and glycans

Our next objective was to see if the aniline additive had an effect on ionization of peptides and to investigate the possibility of simultaneous detection of glycans and peptides. This is important for measuring proteolytic digests of glycoproteins, for concurrent identification of glycosylation sites, and for characterization of site-specific glycan pools, as demonstrated elsewhere[29,30]. A tryptic digest of bovine α_1-acid

glycoprotein was fractionated according to the procedure in the Experimental section and a fraction containing predominantly a single tryptic peptide <Eheeer (a modified qheeer tryptic peptide at *m/z* 810.37, where <E corresponds to pyroglutamic acid) and several sialylated glycoforms of npey**N**k was isolated. A portion of this fraction was treated with PNGase F to partially detach the glycans, and another portion of the fraction was used as a control. The two sets of samples were each analyzed using both DHB and DHB/aniline matrices. Figures 4.5 and 4.6 show the resulting mass spectra. The effect of aniline additive on ionization of peptides is shown in Figure 4.5. For peptides, the DHB matrix yielded clearer spectra (Figure 4.5 A, C) than the DHB/aniline matrix (Figure 4.5 B, D). The latter spectra reveal extensive formation of peptide sodium-adduct ions, implying that aniline either promotes formation of sodiated peptides or that the reagent is contaminated with sodium traces. Overall, the use of DHB/aniline matrix does not appear to enhance, nor does it seem to significantly diminish the signal intensity of peptides. The appearance of a peak at *m/z* 765.35, which corresponds to PNGase F reaction product peptide npey**D**k, indicates that some deglycosylation did take place and that there are free glycans in the PNGase F-treated sample spots.

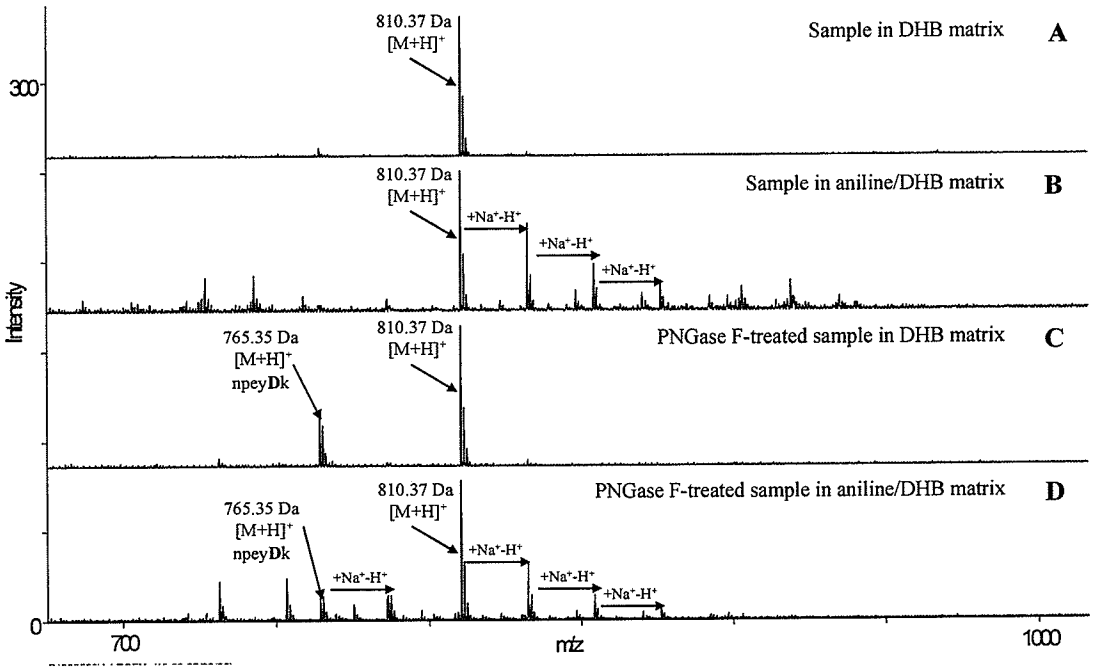


Figure 4.5. The 700-1000 *m/z* region of the MALDI-TOF mass spectra of an LC fraction of a tryptic digest of bovine α_1-acid glycoprotein, containing predominantly <Eheeer tryptic peptide (810.37 Da) and tryptic glycoconjugates of npeyNk. Conditions for each spectrum are given in the figure. <E denotes pyroglutamic acid.

The higher *m/z* region of the same mass spectra, where glycopeptide and glycan peaks are observed, is presented in Figure 6. DHB/aniline matrix appears to hamper ionization of the sialylated glycopeptides (Figure 4.6 B). Lower signal-to-noise ratios, as well as formation of numerous adducts, are evident. The DHB/aniline matrix undoubtedly diminishes the signal quality of the glycopeptides. In Figure 4.7 C, none of the peaks corresponding to free glycans are observed. This is expected, since DHB was already shown to be inferior to DHB/aniline for ionization of oligosaccharides. The sample also contains peptides and glycopeptides, which typically have higher ionization efficiency than glycans[29], and thus may suppress the signal of sugars in the mixture in

these conditions. Figure 4.6 D clearly illustrates the advantage in using DHB/aniline matrix for analysis of oligosaccharides. Single sodium-adduct ions were observed for asialo, and the sialylated glycans produced the same patterns as described for Figure 4.3. Signal intensity of the glycan peaks surpasses that of the glycopeptides. This serves as further evidence for the claim that aniline/DHB matrix preferentially improves the ionization efficiency of glycans.

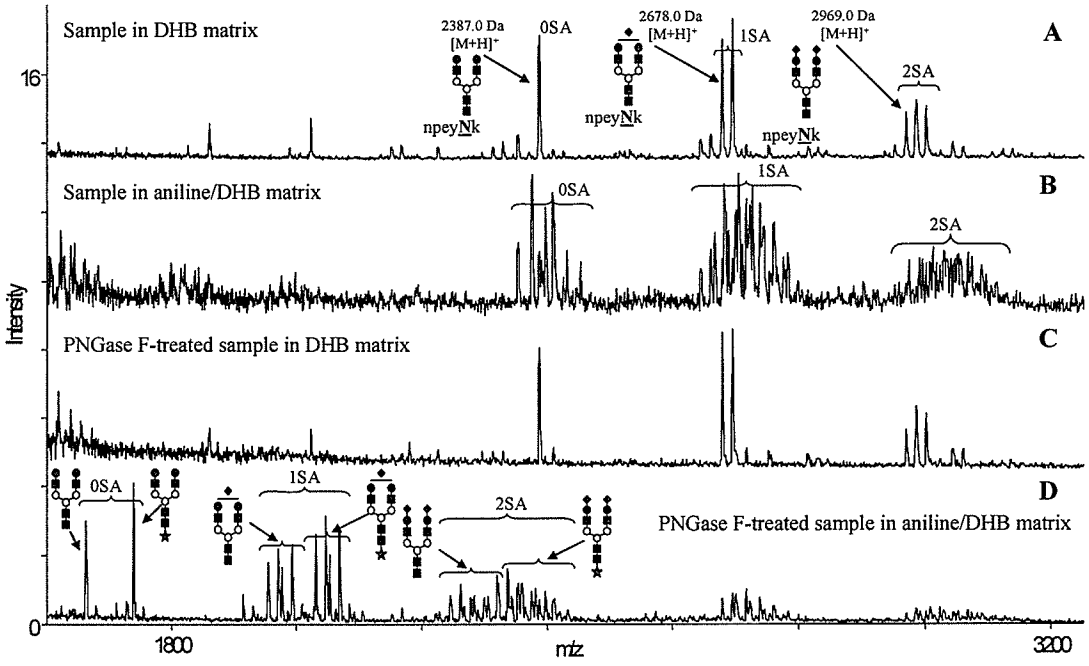


Figure 4.6. The 1600-3200 *m/z* region of the MALDI-TOF mass spectra shown in Figure 4.5. Note that the glycans are observed only where the sample is in aniline/DHB matrix (D). Region labeled 2SA in (D) appears as a composite of region 0SA in (B) and the region shown in Figure 4.3 B.

4.5.6 Glycans from ovalbumin

To probe a different complex glycan mixture, we decided to subject chicken egg white albumin (ovalbumin) glycans to our new method. Ovalbumin is a glycoprotein with a single glycosylation site with over thirty different glycans reported to date[30]. Glycans detached with PNGase F were initially deposited in the DHB matrix, however, very little was detected (not shown). The original sample solution was concentrated by a factor of 10 and analyzed in DHB again. This resulted in sufficient signal to enable structural assignments of the glycans based on the *m/z* values of prominent peaks.

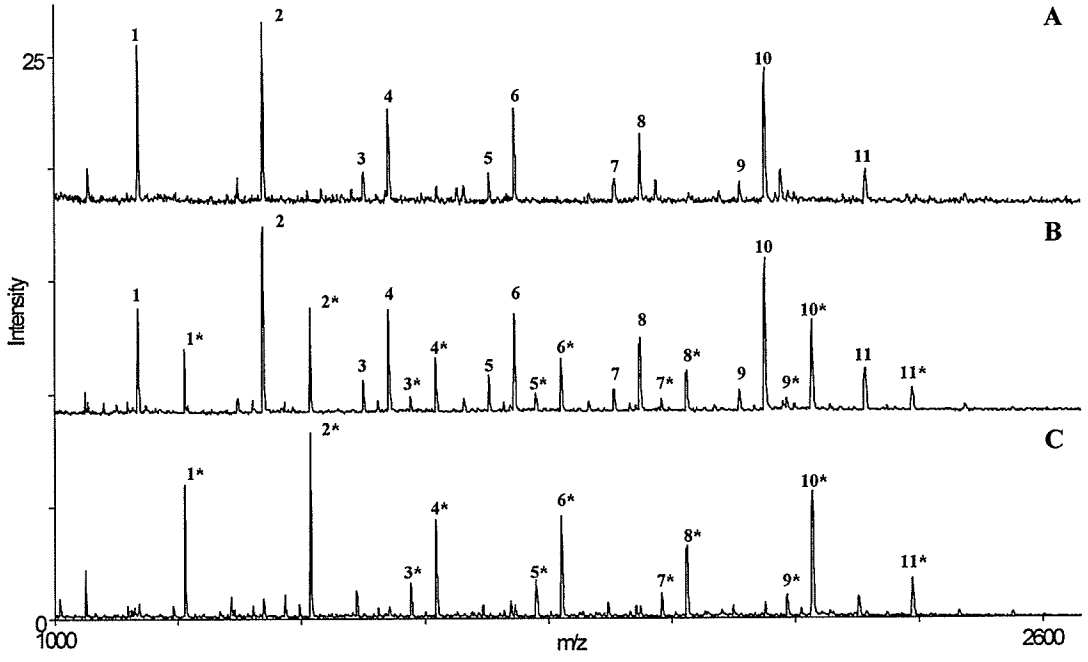


Figure 4.7. MALDI-TOF mass spectra of glycans released from ovalbumin in DHB (A); in aniline: DHB matrix 20 minutes after drying (B); in aniline/DHB matrix 16 hours after drying (C). Structures and masses of the glycans are given in Table 4.2. B and C were acquired from the same sample spot on a MALDI target. Aniline Schiff base form is denoted by an asterisc.

Table 4.2. Selected Ions of Abundant Glycans Released from Ovalbumin and Their Aniline Derivatives.

Glycan number	*m/z* $[M+Na]^+$ (Da)	*Glycan composition*[a]	*Derivative number*	*m/z* $[M^*+Na]^+$ (Da)
1	1136.3	$(HexNAc)_1(Man)_3(GlcNAc)_2$	1*	1211.3
2	1339.3	$(HexNAc)_2(Man)_3(GlcNAc)_2$	2*	1414.3
3	1501.3	$(Hex)_1(HexNAc)_2(Man)_3(GlcNAc)_2$	3*	1576.3
4	1542.3	$(HexNAc)_3(Man)_3(GlcNAc)_2$	4*	1617.3
5	1704.3	$(Hex)_1(HexNAc)_3(Man)_3(GlcNAc)_2$	5*	1779.3
6	1745.3	$(HexNAc)_4(Man)_3(GlcNAc)_2$	6*	1820.3
7	1907.3	$(Hex)_1(HexNAc)_4(Man)_3(GlcNAc)_2$	7*	1982.3
8	1948.3	$(HexNAc)_5(Man)_3(GlcNAc)_2$	8*	2023.3
9	2110.3	$(Hex)_1(HexNAc)_5(Man)_3(GlcNAc)_2$	9*	2185.3
10	2151.3	$(HexNAc)_6(Man)_3(GlcNAc)_2$	10*	2226.3
11	2313.3	$(Hex)_1(HexNAc)_6(Man)_3(GlcNAc)_2$	11*	2388.3

[a]Determined using GlycoMod online tool from ExPASy Proteomics tools.

These glycans are given in Table 4.2, along with their native and aniline-derivative masses. The original (dilute) sample solution was then deposited in DHB/aniline matrix and analyzed. The mass spectra were compared (Figure 4.7 A, B). Although first appearing more complicated than the mass spectrum of glycans in DHB alone, Figure 4.7 B enabled a relatively straightforward assignment of all initially identified glycan peaks and their aniline derivatives. Interestingly, the same sample in DHB/aniline matrix was analyzed again 16 hours later, and most of the glycans had been converted into the aniline-labeled form (Figure 4.7 C). The sample was stored on target under ambient conditions without any further treatment prior to subsequent analysis 16 hours later. This implies that further formation of the Schiff base took place in the solid

phase. It is likely that as water produced in the reaction evaporates from the sample spot, which is essentially the reaction mixture, and since the aniline reagent is in excess, the equilibrium is shifted towards formation of the derivative. Upon making this observation, similar tests, using solutions with glycans from human and bovine α_1-acid glycoproteins, were performed. Results showed that the same trend, although we were unable to observe complete elimination of the native glycan signals (data not shown). Nevertheless, strong evidence for solid phase reaction was apparent in all sets of samples. As noted in Figure 4.2, the abundance ratios of derivatized/native forms were relatively constant, making this method applicable to semi-quantitative analyses. Analogous solid-phase reactions have been previously reported elsewhere[32]. A more thorough investigation of this interesting phenomenon in the context of MALDI is currently under way.

4.6 Conclusions

This study demonstrates a novel approach of delivery and application of aniline as a reagent for non-reductive amination of oligosaccharides in analyses by MALDI mass spectrometry. Aniline was shown to act both as an effective derivitization agent for oligosaccharides, producing a stable Schiff base derivative, and a useful DHB matrix additive for improving ionization of glycans. The reaction takes place upon depositing and mixing the sample on-target with DHB matrix containing aniline, concurrently with the drying of the spot in a matter of minutes. The use of our novel matrix resulted in formation of finer matrix/sample crystals on polished steel targets relative to those formed by unmodified DHB, leading to a more uniform sample distribution within a spot. This eliminates the need to look for a "sweet spot" to obtain adequate signal for the

analyte. Both aniline-derivatized and native glycans showed intense signals in the mass spectra. This in-tandem occurrence allows for an additional means of identification of glycans in the mass spectrum based on the derivative mass and native masses, thus aiding in peak assignment. This method also discriminates between glycans and peptides, as peptides were not significantly affected by our derivatization procedure. MS/MS spectra of derivatized oligosaccharides contained predominantly Y- and B-ions, making possible preliminary structural elucidation of a given glycan. The observed time-dependence of the derivatization reaction in the solid phase may also be utilized effectively for identification of free reducing-end oligosaccharides in biological samples.

4.7 Acknowledgements

The authors thank Dr. Oleg Krokhin for technical assistance and Drs. K. G. Standing and W. Ens for the use of their mass spectrometer. The Natural Sciences and Engineering Council of Canada (NSERC), the Canada Research Chairs program (CRC) and the Canadian Foundation for Innovation (CFI) are acknowledged for funding. The authors also wish to thank Drs. Julian Saba, Mark McComb, Martin Ethier, David Perlman, Gro Thorne-Tjomsland, and Maureen Spearman for helpful discussions.

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Chapter 5:

Investigation of use of *N,N*-dimethylaniline as MALDI matrix additive for analysis of oligosaccharides by MALDI MS.

5.1 Author's Contributions

All experiments pertaining to this work were designed and conducted by Sergei I. Snovida. All figures and manuscript drafts were also created by Sergei I. Snovida. Dr. H. Perreault was responsible for editing the final version of the manuscript prior to initial submission to Rapid Communications in Mass Spectrometry journal and providing funding for this research. Both authors contributed equally to final revision of the manuscript prior to its publication.

A 2,5-Dihydroxybenzoic Acid/*N,N*-Dimethylaniline Matrix for the Analysis of Oligosaccharides by Matrix-Assisted Laser Desorption Ionization Mass Spectrometry.

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Published: *Rapid Commun. Mass Spectrom.* **2007**, *21*, 3711-3715

5.2 Abstract

The use of a novel 2,5-dihydroxibenzoic acid/*N,N*-dimethylaniline (DHB/DMA) matrix-assisted laser desorption/ionization (MALDI) matrix for detection and quantitative analysis of native *N*-linked oligosaccharides was investigated in this study. Substantial improvements in sensitivity were observed relative to the signals obtained with a traditional DHB matrix. Moreover, the morphology of the matrix crystal layer was very uniform, unlike that of DHB. This resulted in highly homogeneous sample distribution throughout the spot, allowing reproducible and consistent mass spectra to be obtained without spot-to-spot variations in signal. Here, we also demonstrate an approach for performing sensitive and accurate quantitative analysis of native *N*-linked glycans with this novel matrix using an internal standard method.

5.3 Introduction

Glycosylation is among the most common forms of post-translational modification in proteins. It is involved in a variety of biological processes, including protein folding, signaling, and cell-cell interactions[1,2]. The ability to fully characterize a given glycoprotein in terms of glycosylation sites, glycan structures, site occupancy and extent of heterogeneity in the population of glycan structures can offer a better insight into mechanisms of the biological events which a given glycoprotein is involved. This information may also be used as a predictive tool to detect and identify the causes of abnormalities in the physiological state of an organism, as changes in glycosylation patterns may often indicate a disease state or a genetic defect.

Mass spectrometry (MS) is a very sensitive analytical tool available to the research community today. Matrix-assisted laser desorption ionization mass spectrometry (MALDI MS), typically with a time-of-flight (TOF) tube as a mass analyzer, is used extensively for analysis of glycoprotein-derived oligosaccharides. It offers several advantages over other MS techniques, mainly in terms of sample size requirements, relative simplicity of spectral interpretation, and possibility of sample archiving for later use.

Perhaps the most challenging aspect of analysis of oligosaccharides by MALDI MS is the somewhat low ionization efficiency of carbohydrates in the gas phase[3]. As the signal intensity in the mass spectrum is related to the number of analyte ions in the gas phase generated during a MALDI event, sensitive analysis of native oligosaccharides has been difficult relative to the characterization of easier ionized compounds, e.g. peptides[4]. Derivatization of oligosaccharides, generally by permethylation, reductive amination, or

through the formation of a stable Schiff base, addresses this problem by improving ionization of carbohydrates[5-7]. Most derivatization procedures, however, still require fairly large amounts of sample and due to the number and nature of sample handling steps involved, these methods are generally susceptible to sample loss and contamination[3,4].

We have recently reported on the use of 2,5-dihydroxybenzoic acid/aniline (DHB/An) matrix for analysis of native oligosaccharides[8]. Intense signals for both the Schiff base aniline derivatives and the native glycans were observed in the MALDI-TOF mass spectra. In the present work we demonstrate the use of a similar hybrid matrix, 2,5-dihydroxybenzoic acid/*N,N*-dimethylaniline (DHB/DMA), for the analysis of native *N*-linked glycans. This matrix offers significant improvements over the classical DHB, which is the most widely cited MALDI matrix for analysis of carbohydrates. It is also superior to our recently reported DHB/An matrix. The benefits of using DMA as an additive to α-cyano-4-hydroxycinnamic acid (CHCA) for the analysis of peptides has been reported elsewhere[9]. We investigated the potential of the new DHB/DMA matrix for the quantitative analysis of native oligosaccharides.

5.4 Experimental Section

5.4.1 Materials and reagents

Ovalbumin (chicken egg white albumin), maltohexaose, maltoheptaose, dextran standard 1000, ammonium bicarbonate, and 2,5-dihydroxybenzoic acid were obtained from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile (ACN), glacial acetic acid (TraceMetal grade), and reagent grade *N,N*-dimethylaniline were purchased from Fischer

Scientific (Fair Lawn, NJ, USA). PNGase F endoglycosidase enzyme was purchased from ProZyme (San Leandro, CA, USA). Milli-Q water was used in preparation of all solutions.

5.4.2 Sample preparation

Standard solutions were obtained by dissolving 2 mg of each standard (maltohexaose, maltoheptaose, and dextran standard 1000) in 1 mL of water. The solution was mixed using an agitator for 30 seconds to ensure complete dissolution. A series of thirteen 1 in 2 dilutions were carried out by mixing 0.5 mL of the initial standard solution with 0.5 mL of water, and repeating this step with the new solution. Thus, a total of fourteen standard solutions, ranging in concentration from about 0.2 ng/μL to 2 μg/μL of the material, were obtained for each set.

Ovalbumin oligosaccharides were obtained by first dissolving 2 mg of ovalbumin in 200 μL of 25 mM ammonium bicarbonate buffer at pH 7.8 in a 0.6 mL microcentrifuge tube. PNGase enzyme solution (5 μL, as provided by the manufacturer) was added to the tube and the contents were mixed using an agitator for about one minute. The mixture was incubated at 37 °C for 20 hours, with additional mixing after the first 10 hours. Detached glycans were isolated by loading 50 μL of the digestion mixture onto a C_{18} reverse-phase (RP) column, which was prepared by a previously described procedure[10] and flushing it with 200 μL of water to elute the sugars. The eluted sugar solution was mixed to ensure homogeneity in concentration.

5.4.3 MALDI matrix preparation

DHB matrix solution was prepared by dissolving 100 mg of DHB in 1 mL of a 1:1 solution of water and ACN. The DHB/DMA matrix solution was prepared by adding 20 μL of neat *N,N*-dimethylaniline to the DHB matrix solution. *N,N*-dimethylaniline was purified by simple distillation prior to use. Samples were deposited onto a polished steel MALDI target by mixing the analyte and matrix solutions (1 μL each) on-target and allowing the mixture to dry by evaporation. All DHB matrix/sample co-crystals were re-dissolved in ethanol to obtain more uniform layers upon re-crystallization. DHB/DMA spots were left untreated after deposition and crystallization.

5.4.4 Mass spectrometric measurements.

All mass spectra were acquired on a Bruker Biflex IV MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) in the positive ion reflecting mode.

5.5 Results and discussion

5.5.1 Preliminary work

Our goal in this study was to examine the efficacy of various aniline derivatives for use as DHB matrix additives for the analysis of native oligosaccharides. In our previous work[8] we had observed substantially enhanced signals for native sugars in the MALDI mass spectra acquired in a DHB/An matrix compared to unmodified DHB. However, strong signals of aniline Schiff base derivative ions were also abundant in these spectra. Native oligosaccharides, including their Schiff base derivatives, ionized as sodiated ions. Despite the significant increase in signal intensity, manual spectral interpretation was

made more difficult. It is generally taken for granted that derivatization of oligosaccharides is necessary for improving their signal intensity in MALDI spectra; however, our results suggest that other factors related to the physicochemical properties of matrix materials may be responsible for the differences in observed inherent ionization efficiencies of oligosaccharides in various MALDI matrices.

One of the first selections was *N,N*-dimethylaniline (DMA), a compound in which the *N*- hydrogens are replaced by two methyl groups. As it is a tertiary amine, DMA is unable to form a Schiff base with the reducing end of an oligosaccharide, unlike aniline. Due to their structural resemblance, both compounds were expected to interact similarly with DHB and thus produce a similar effect in enhancing the ionization efficiency of native sugars (as observed with DHB/An matrix), however without formation of the Schiff base derivatives.

The notion that sample material is not evenly distributed within a DHB spot deposited by a conventional dried droplet technique is well established[11]. Sample mixture components with similar properties are usually segregated and form what is known as "sweet spots". This is typically attributed to several factors, including the uneven, needle like crystal formations of DHB material created by this method, differences in solubility of the sample components in the matrix and sample solvents, differences in analyte affinity for matrix material, and uneven drying of a sample/matrix mixture. There are several ways in which this phenomenon could be minimized, including deposition of very small diameter sample spots (typically on AnchorChip™ or similar targets with hydrophobic coatings), re-dissolving a sample spot in cold ethanol or acetone to obtain a

more uniform crystal layer, or more recently, by using ionic liquid matrices to improve analyte distribution[12,13].

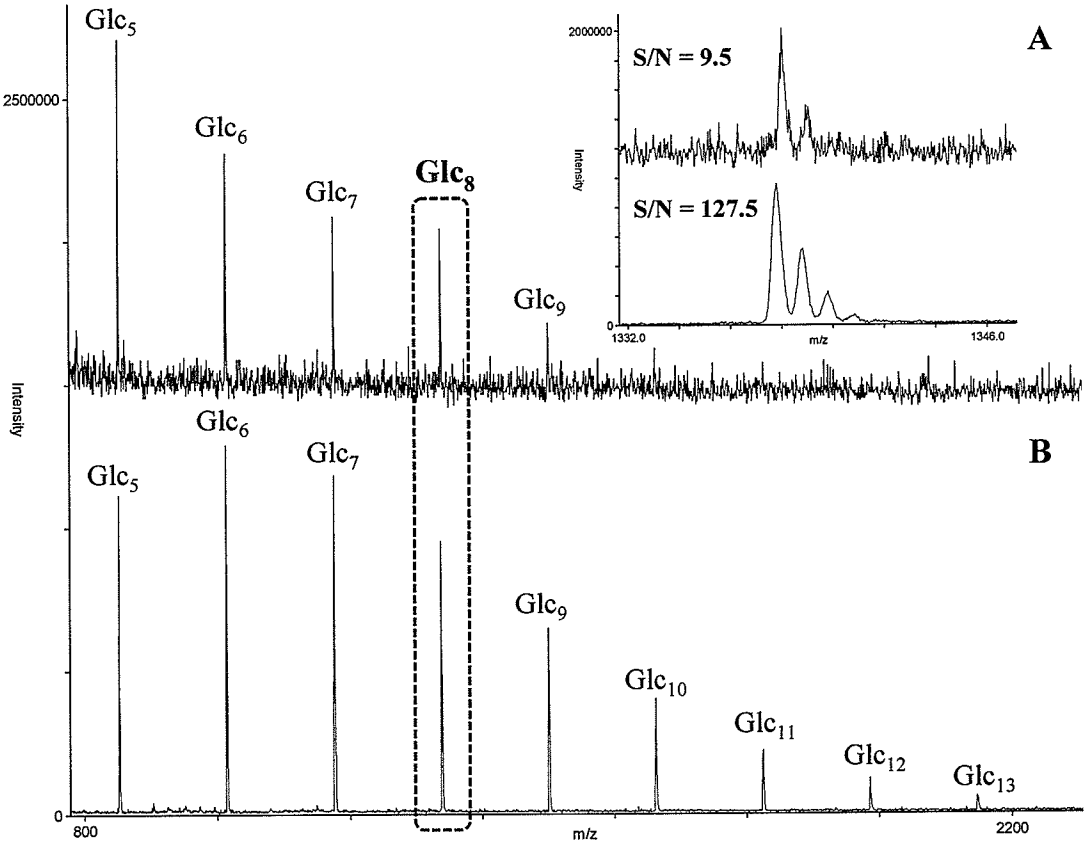

Figure 5.1. Positive ion reflecting mode axial MALDI-TOF mass spectra of Dextran 1000 standard in DHB (A) and in DHB/DMA (B). Inset in (A) compares signal-to-noise ratio of Glc_8 peaks between mass spectra acquired in DHB (top) and DHB/DMA (bottom). About 25 ng of Dextran 1000 material were deposited per spot.

We employed sample re-dissolution method to obtain a uniform morphology of a DHB spot with about 25 ng of Dextran 1000 standard (a mixture of glucose polymers with a number average molecular weight (M_n) of about 1000 Da). For comparison, the

same amount of sample was mixed on-target with DHB/DMA matrix solution and was allowed to dry. The morphology of the DHB/DMA crystal layer was similar to that obtained with DHB/An matrix[8]. The corresponding mass spectra are given in Figure 5.1. Oligosaccharides were observed as sodiated ions. A vast improvement in mass spectral quality in terms of signal-to-noise ratios for native carbohydrates is evident in Figure 5.1 B (spectrum acquired in DHB/DMA) relative to Figure 5.1 A (DHB). A high degree of spectral reproducibility was obtained with DHB/DMA in contrast to DHB which, used alone, yielded drastic variations in peak relative heights depending on the location sampled. Moreover, the peak average molecular weight M_p for the mixture calculated from the spectrum in Figure 5.1 B on its entire *m/z* range (not shown) and repeated spectra was consistently similar to the M_n value indicated on the manufacturer's certificate of analysis (determined by gel-permeation chromatography and end-group titration analysis).

5.5.2 Quantitative analysis of glycans by MALDI MS

This important improvement in signal intensity and reproducibility with DHB/DMA relative to DHB suggested the possibility of developing an accurate and sensitive quantitative method for the analysis of oligosaccharides using this new matrix material. Among quantitative methods recently developed for MALDI MS of carbohydrates, isotopic labeling of by permethylation[13] is a very good way to perform relative quantitation. While offering several unique advantages, the technique ultimately relies on derivatization of the native glycans to obtain adequate signal and requires sample homogeneity in the matrix to enable relative quantitation. We decided to explore

the use of our new DHB/DMA matrix for quantitative analysis. To test this possibility, a series of mixed maltooligosaccharide standard solutions were prepared. The concentration of maltohexaose was held constant at about 6.3 pmol/μL as internal standard and that of maltoheptaose ranged between about 0.7 to 22.4 pmol/μL. The mixtures were deposited on a target in triplicates with DHB/DMA matrix. The corresponding mass spectra are given in Figure 5.2.

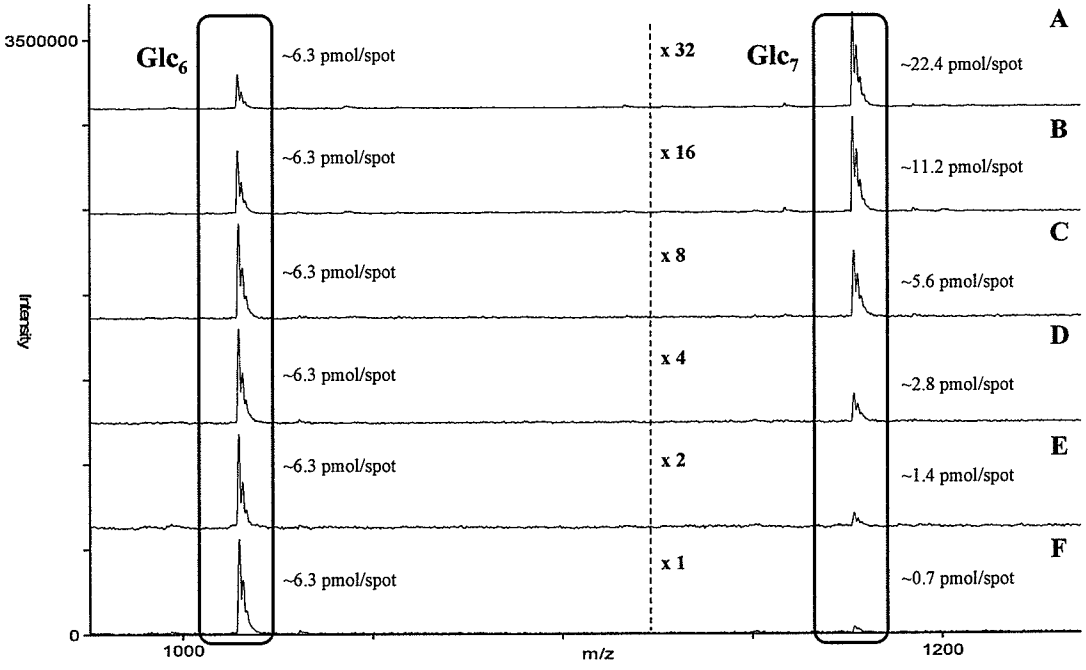

Figure 5.2. Positive ion reflecting mode axial MALDI-TOF mass spectra of maltohexaose (Glc_6) and maltoheptaose (Glc_7) in DHB/DMA matrix. Same amount of maltohexaose is present in each spot (~6.3 pmol). Amount of maltoheptaose per spot is varied from ~7 pmol to ~22.4 pmol.

If uniform sample distribution yields raw signal intensities that depend on the number of laser shots fired, then measurements should be based on a same number of

shots for each sample. This was observed for maltoheptaose and maltohexaose, which independently produced the same signal intensity vs. concentration relationship. However, in order to make the measurements more accurate and consistent for a given analyte, the signal intensity relative to that of a standard should be used, so that the response becomes independent of the number of laser pulses. Here, the signal intensity of maltoheptaose (analyte) relative to that of maltohexaose (internal standard) was used as the response. Signal intensity was obtained by calculating the sum of areas of all peaks in the isotopic envelope pertaining to a particular compound. A linear response was obtained for the concentration range investigated (Figure 5.3). These results indicated that, in this given range, quantitative analysis of neutral oligosaccharides by MALDI MS is feasible at the femtomole level using the DHB/DMA matrix.

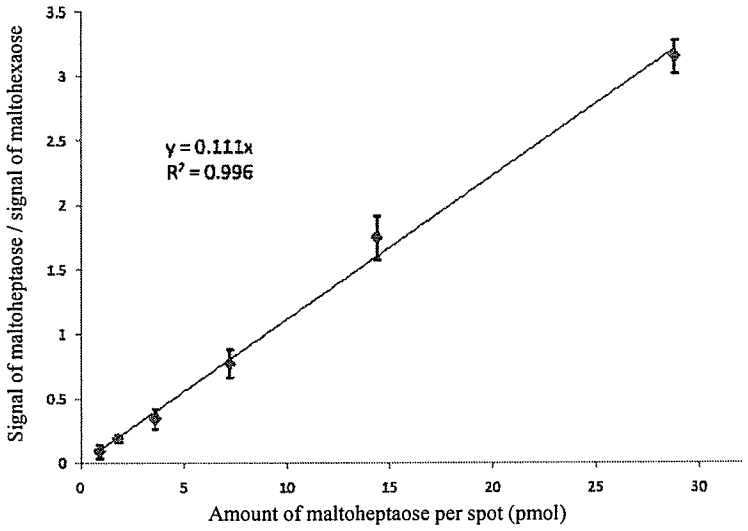

Figure 5.3. Relative response of varying amounts of maltoheptaose to maltohexaose (internal standard) from mass spectra in Figure 5.2.

The next step in evaluating the technique involved oligosaccharides obtained from digestion of chicken ovalbumin with PNGase F glycosidase. Based on the amount of glycoprotein used and assuming complete enzymatic deglycosylation, several ovalbumin sugar solutions were prepared, at concentrations estimated from ca. 2 to 20 pmol/μL. Maltohexaose was used as an internal standard. It was added to all solutions, so that each contained ~6.3 pmol/μL of the standard. DHB/DMA was used as the matrix and samples were spotted in triplicates. The mass spectra of the mixtures are given in Figure 5.4. Several qualitative features are from the spectra: the relative signal intensities of ovalbumin glycans remain constant throughout the experiments and their signal intensities do appear to vary in proportion to the amount of the material deposited relative to the internal standard. Two $[M+Na]^+$ oligosaccharide peaks at *m/z* 1135.3 and 2312.4 were selected because of (i) the large difference in their relative signal intensities, and (ii) they are far apart in the *m/z* range covered by the majority of glycan peaks. The linear dependence on concentration for the two glycans is illustrated in Figure 5.5, where data points are reported as (ovalbumin glycan)/(maltohexaose) intensity ratios.

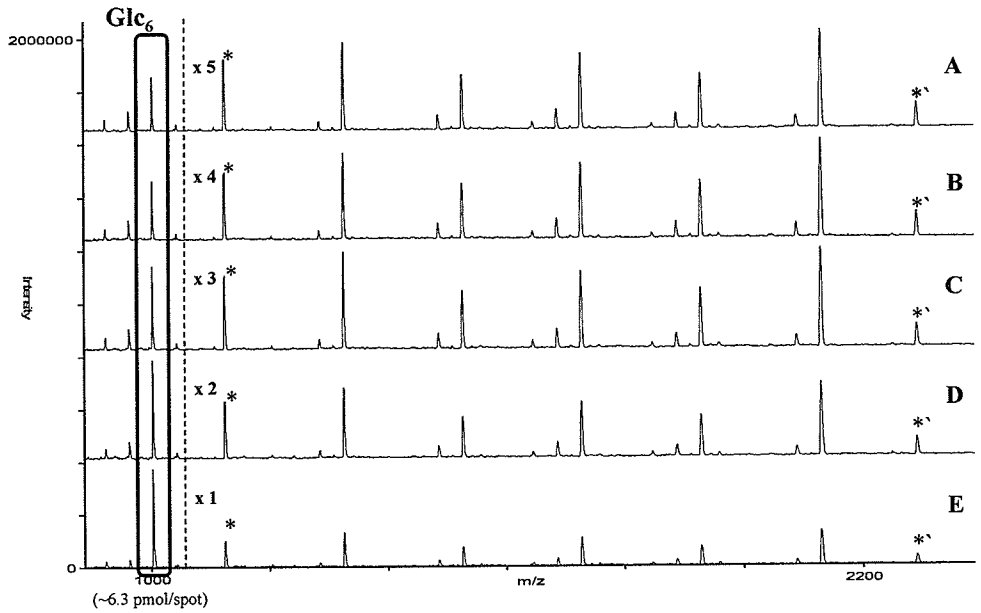

Figure 5.4. Positive ion reflecting mode axial MALDI-TOF mass spectra of chicken ovalbumin glycans. Maltohexaose is used as internal standard. Note that the relative peak intensities within the glycan pool remain the same within the analysis range. Peaks designated by * and *` correspond to glycans at 1135.3 *m/z* and 2312.4 *m/z*, respectively.

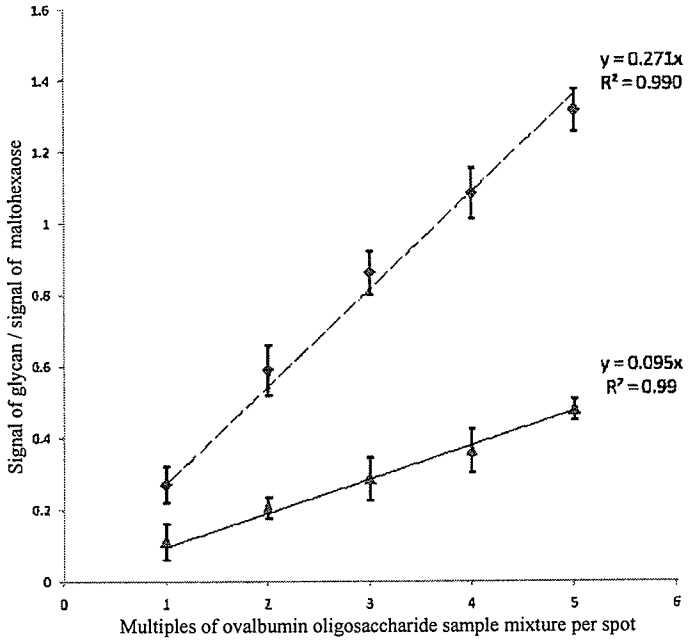

Figure 5.5. Relative response of varying amounts of ovalbumin glycans at 1135.3 *m/z* (♦) and 2312.4 *m/z* (▲) to maltohexaose (internal standard) from Figure 4.

This illustrates, as a proof of concept, that the method may be employed for quantitative analysis of complex multi-component oligosaccharide mixtures with the sub pmol/μL range per component. Uncertainties may be minimized by increasing the number of sample replicates and measurements. One critical assumption made in this method is that ionization efficiencies of all neutral oligosaccharide species studied in this work are identical. This may not be true for all oligosaccharides; however, there is convincing evidence to support this assumption, i.e. very little variation in the ionization efficiencies of neutral glycans in the 1000-3000 Da range[2]. Also, the experiments leading to Figure 5.5 were conducted reversely, that is keeping constant the concentration of ovalbumin glycans while varying the amount of internal standard (not shown). The responses observed for *m/z* 1135.3 and 2312.4 relative to maltohexaose were similar.

Although an ideal internal standard for a given analyte is its isotopically labeled analog, incorporating such a standards for each glycan studied would be extremely costly. The procedure presented here, involving the use of DHB/DMA matrix, employs relatively inexpensive maltooligosaccharides as internal standards. Moreover, considering the small sample amounts required and rapid sample preparation procedures involved, this method is very efficient for relative quantitation of neutral oligosaccharides and may be used to estimate the absolute quantities of neutral oligosaccharides in a sample.

5.6 Conclusions

This study has demonstrated the application of a novel DHB/DMA matrix for analysis of native oligosaccharides at the femtomole level without the need for

derivatization to improve signal quality or isotopic labeling for quantitative analysis. This new technique offers a quick, simple, and sensitive approach for analyzing neutral carbohydrates.

5.7 Acknowledgements

The authors would like to thank The Natural Sciences and Engineering Council of Canada (NSERC), the Canada Research Chairs program (CRC) and the Canadian Foundation for Innovation (CFI) for funding. The authors also wish to acknowledge Colin Lee and Vincent C. Chen for their invaluable technical assistance and helpful discussions.

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Chapter 6:

Development of a method for automated identification and quantitative analysis of oligosaccharides by MALDI MS.

6.1 Authors Contributions

All experiments pertaining to this work were designed and conducted by Sergei I. Snovida. Justin M. Rak-Banville was responsible for writing the algorithm for identification of glycans under the direction of Sergei I. Snovida. All figures and manuscript drafts were created by Sergei I. Snovida. Dr. H. Perreault was responsible for editing the final version of the manuscript prior to initial submission to the Journal of American Society for Mass Spectrometry and providing the funds pertaining to this research. All authors contributed equally to final revision of the manuscript prior to its publication.

On the Use of DHB/Aniline and DHB/*N,N*-dimethylaniline Matrices for Improved Detection of Carbohydrates: Automated Identification of Oligosaccharides and Quantitative Analysis of Sialylated Glycans by MALDI-TOF Mass Spectrometry

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Published: *J. Am. Soc. Mass Specrom.* **2008**, *19* (8), 1138-1146

6.2 Abstract

In this study we demonstrate the application of 2,5-dihydrohybenzoic acid/aniline (DHB/An) and 2,5-dihydroxybenzoic acid/*N,N*-dimethylaniline (DHB/DMA) matrices for automated identification and quantitative analysis of native oligosaccharides by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Both matrices are shown to be superior to pure DHB for native glycans in terms of signal intensities of analytes and homogeneity of sample distribution throughout the crystal layer. On-target formation of stable aniline Schiff base derivatives of glycans in DHB/An and complete absence of such products in the mass spectra acquired in DHB/DMA matrix, provide a platform for automated identification of reducing oligosaccharides in the MALDI mass spectra of complex samples. We also show how enhanced sensitivity is achieved with the use of these matrices and how the homogeneity of deposited sample material may be exploited for quick and accurate quantitative analysis of native glycan mixtures containing neutral and sialylated oligosaccharides in the low picomole to mid femtomole range.

6.3 Introduction

Carbohydrates are widely recognized today for their vital roles in the biology of organisms[1-4]. They exist as free oligosaccharides or as elements of more complex conjugated systems. Glycosylation is an important form of post-translational modification of proteins. Diverse processes, including cell adhesion, protein folding, immune response, signaling and trafficking, are only some of the important events in which these biomolecules are implicated. In recent years, it has become more apparent that protein glycosylation is ubiquitous, and it is estimated that a significant proportion of all proteins, including viral and bacterial proteins, bear this modification[5,6]. One may thus obtain a better insight into the biological role and mechanism of a given glycoprotein by identifying its glycosylation sites and characterizing them in terms of degree of occupancy, oligosaccharide structures, and population heterogeneity of the glycan structures found. As changes in glycosylation patterns may be used as biomarkers, generated datasets may be used as predictive or diagnostic tools for detection and identification of biochemical abnormalities, which may be correlated to any pathology observed in a given organism[1,7,8].

As a result, there is an increasing need for developing efficient methods for detection and structural characterization of this important class of biomolecules. Among many complementary technologies for the analysis of biological carbohydrates available today, mass spectrometry (MS) has emerged as a key tool at the forefront of glycobiology-related research. It provides efficient and sensitive means for studying carbohydrates in the low concentration clinical-scale samples, and may also be effectively coupled to various separation techniques, both on- and off-line[9,10].

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has proven itself as an extremely useful technique for the analysis of various biomolecules[11,12]. MALDI offers several advantages over other ionization techniques, mainly in terms of modest sample size requirements, relative simplicity of spectral interpretation, and possibility of sample archiving for later use. There still exist some challenges in the analysis of oligosaccharides by MALDI-MS, which generally stem from the inherently low ionization efficiency of carbohydrates relative to peptides and proteins[9]. One solution to this problem is to operate an instrument at higher laser power, thus increasing the number of ions produced per MALDI event. This, however, often leads to more extensive fragmentation of the analytes, especially if labile groups such as sulfates or sialic acids are present. A decrease in the signal-to-noise ratio due to increased formation of the matrix cluster ions[13] would also be observed as a result. Hence the mass spectra obtained do not provide a true representation of the sample constituents and the signal quality is generally diminished. It is therefore difficult to obtain sensitive analysis of native oligosaccharides compared to other compounds such as peptides, for example.

Oligosaccharides are often derivatized prior to MALDI MS in order to remedy this problem. Most derivatization procedures involve chemical modification of the glycans by permethylation, reductive amination, or stable Schiff base formation to render the analytes more susceptible to ionization[9,14-16]. Although derivatized forms bring substantial improvements in sensitivity relative to native forms, these methods are often susceptible to sample loss and contamination.

Our recent publications[17-19] have demonstrated how the incorporation of aniline (An) and *N,N*-dimethylaniline (DMA) into a conventional 2,5-dihydrohybenzoic acid matrix solution results in significant improvement in sensitivity for native oligosaccharides when analyzed by MALDI-TOF MS. The present work describes how the two matrices may be used in tandem for automated identification of glycans in mass spectra of complex mixtures. The discussion is also extended the application of DHB/DMA matrix for quantitative analysis of native and sialylated glycans in the positive-ion extraction mode.

6.4 Experimental

6.4.1 Materials and reagents

Ovalbumin (chicken egg white), α_1-acid glycoprotein (human), maltohexaose, maltoheptaose, trifluoroacetic acid (TFA), ammonium bicarbonate, and 2,5-dihydroxybenzoic acid (DHB) were obtained from Sigma (St. Louis, MO, USA). HPLC grade acetonitrile (ACN), reagent grade aniline (An) and *N,N*-dimethylaniline (DMA) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). PNGase F endoglycosidase enzyme was purchased from ProZyme (San Leandro, CA, USA). Milli-Q water was used in preparation of all solutions.

6.4.2 Sample preparation

Standard solutions were obtained by dissolving 2 mg of each standard (maltohexaose and maltoheptaose) in 1 mL of water. Each solution was mixed using an agitator for 30 seconds to ensure complete dissolution. A series of thirteen 1-in-2

dilutions were carried out by mixing 0.5 mL of the initial standard solution with 0.5 mL of water, and repeating this step with the new solution. Thus, a total of fourteen standard solutions, ranging in concentration from about 0.2 ng/μL to 2 μg/μL of the material were obtained for each set.

Ovalbumin and α_1-acid glycoprotein oligosaccharides were obtained by first dissolving 2 mg of protein in 200 μL of 25 mM ammonium bicarbonate buffer at pH 7.8 in a 0.6 mL microcentrifuge tube. PNGase enzyme solution (2 μL, as provided by the manufacturer) was added to the tube and the contents were mixed using an agitator for about one minute. The mixture was incubated at 37 °C for 20 hours, with additional mixing after the first 10 hours. Upon digestion, the mixture was lyophilized and re-suspended in 200 μL of water. Detached glycans were isolated by loading 50 μL of the digestion mixture onto a C_{18} reversed-phase column, which was prepared by a previously described procedure[20] and flushing with 200 μL of 0.5% acetic acid aqueous solution to elute the sugars. The eluted sugar solution was then incubated at 37 °C for 2 hours in order to fully de-aminate the glycans. It was then lyophilized, re-suspended in 200 μL of water, and mixed to ensure homogeneity.

The solution containing the glycans detached from α_1-acid glycoprotein was divided into three portions of 50 μL each. One sample was left untouched (untreated). To another sample (partially desialylated), 50 μL of 0.5% TFA in water were added and the resulting solution was heated at 75 °C for 15 minutes to partially remove sialic acid residues from the glycans. The third sample (fully desialylated) was also treated with TFA as above, although the solution was incubated at 75 °C for one hour to ensure complete removal of sialic acid from the oligosaccharides in the sample. Solutions treated

with TFA were evaporated on a SpeedVac (ThermoScientific, Milford, MA, USA) system and the samples were re-suspended in 50 μL of water.

6.4.3 MALDI matrix preparation

DHB matrix solution was prepared by dissolving 100 mg of DHB in 1 mL of a 1:1 solution of water and ACN. The DHB/DMA matrix solution was prepared by adding 20 μL of neat An or DMA to the DHB matrix solution. Thus, the molar ratio of DHB to An or DMA was approximately 3:1. Both An and DMA were purified by simple distillation prior to use. Samples were deposited onto a polished steel MALDI target by mixing the analyte and matrix solutions (1 μL each) on-target and allowing the mixture to dry by evaporation under ambient conditions.

6.4.4 Mass spectrometric measurements

All mass spectra were acquired on a Bruker Biflex IV MALDI-TOF, an axial TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA) equipped with a 337 nm laser. Mass spectra were obtained in the positive ion extraction mode with the following voltage settings: ions source 1 (19.0 kV), ion source 2 (15.90 kV), and lens (9.3 kV). The reflector voltage was set to 20 kV. The laser was pulsed at 7 Hz and pulsed ion extraction time was set to 400 ns. Laser power was kept in the 20-25% range, at a level only slightly above the threshold for obtaining a good intensity signal without significant shifts in the baseline or introduction of extensive noise into the mass spectra. This is explained further in Results and Discussion. For analyses of the samples using DHB/An and DHB/DMA matrices, the spots of the deposited material were sampled at five random locations (50

laser pulses each) and the signals were added together. In the cases where DHB was used as the matrix, mass spectra were collected at "sweet spots" and taken as the average of 250 laser shots. Where it was not possible to manually identify a "sweet spot", a "five random locations" approach, as described above, was adopted. All oligosaccharides observed in the mass spectra corresponded to $[M+Na]^+$ ions.

6.5 Results and discussion

6.5.1 Preliminary work

Because 2,5-dihydroxybenzoic acid (DHB) is the most widely used MALDI matrix in the literature for analysis of oligosaccharides by MALDI-MS, it was used as a benchmark for testing the performance of the DHB/An and DHB/DMA mixtures as MALDI matrices. As reported earlier[18,19], both DHB/An and DHB/DMA offered impressive improvements in sensitivity for native glycans observed as sodiated ions compared to unmodified DHB as a matrix.

Morphological differences in the crystal layers resulting from sample deposition in DHB and DHB/DMA by the dried droplet technique are seen in Figure 6.1 (A and B). DHB produces irregular needle-shaped crystals, whereas the use of DHB/DMA (and DHB/An also) leads to formation of a more uniform, fine crystal layer. It was possible to detect signals of native glycans as $[M+Na]^+$ ions in the low-picogram range using these matrices, as seen in Figure 6.1 C. The relationship between morphology of matrix crystal layer and analyte sensitivity has been well established: generally, more uniform crystal layers result in more homogeneous sample distribution within a spot, thus making detection of the analyte more even throughout the spot[21,22]. A common technique used to

improve sensitivity with DHB as well as other matrices yielding irregular crystal morphologies is to re-dissolve the spot on-target in cold ethanol or acetone. In this study, redissolving DHB sample spots in cold ethanol did enhance crystal homogeneity and intensity of signals observed, although significantly less than with the DHB/An or DHB/DMA preparation method[18,19]. Moreover, the redissolving procedure appeared useful only if a spot already contained at least 10 pmol (~20 ng) of sample material.

6.5.2 Automated identification of glycans

The use of DHB/An matrix results in the formation of an aniline Schiff base at the reducing end of the native oligosaccharides, as indicated by the presence of the peaks labeled with asterisks in Figure 6.2 B (glycans detached from chicken ovalbumin). This on-target derivatization reaction does not go to completion, and strong signals corresponding to the underivatized native glycans are also observed in the mass spectrum. Although there was a certain time dependence on the extent of derivatization (the Schiff base signal does become stronger with time)[17], about 5 hours after depositing the sample on target, signal intensities of the native and the Schiff base forms of the sample glycans in low-picomole (nanograms) quantities were comparable. Thus, the only differences in the mass spectra of oligosaccharides in DHB/An and DHB/DMA matrices are the peaks labeled with asterisks that correspond to the Schiff base derivatives when DHB/An was used.

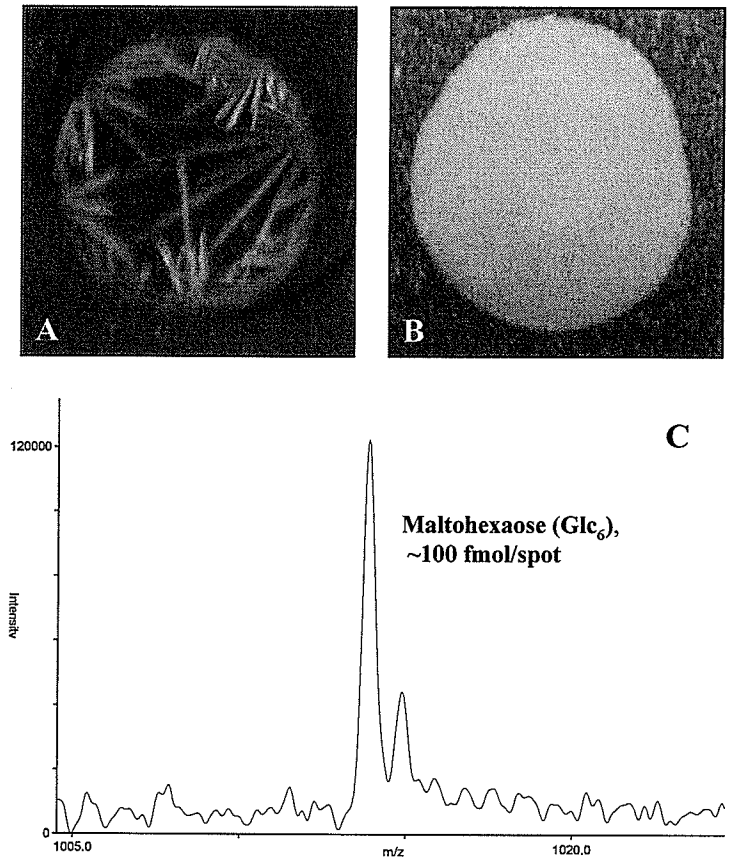


Figure 6.1. Matrix crystal morphologies: favorable properties of DHB/An and DHB/DMA MALDI matrices for the analysis of oligosaccharide mixtures. Sample spots on a polished steel surface, DHB (A) and DHB/DMA (B) obtained by the dried droplet deposition method. (C) Signal due to ~100 fmol of maltohexaose as $[M+Na]^+$ ion in DHB/DMA in reflecting mode MALDI.

There was no significant improvement in ionization efficiency of the Schiff base relative to the native glycans. DHB/An thus appears as a less useful MALDI matrix than DHB/DMA, mainly because the signal attributed to a particular glycan is split into two peaks. This affects the sensitivity and yields mass spectra that are more complex for interpretation.

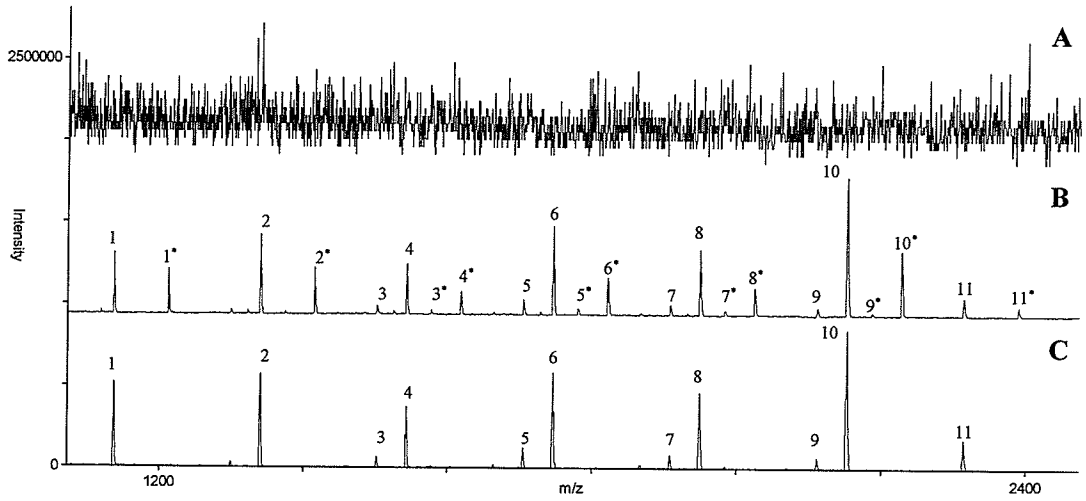
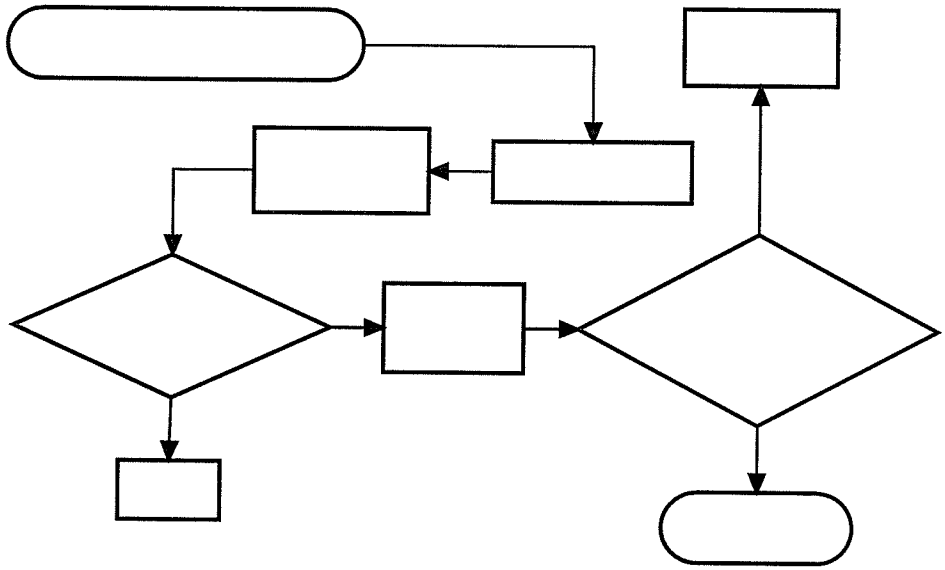


Figure 6.2. Reflecting mode MALDI-TOF mass spectra of oligosaccharides released from chicken ovalbumin by PNGase F (~ 5 μg digest solution) in DHB (A), DHB/An (B), and DHB/DMA (C). Prominent glycan peaks are labeled and aniline Schiff base derivatives are denoted by an asterisk. All glycans were observed as $[M+Na]^+$ ions. Note that no signal was detected in the DHB sample spot.



Scheme 6.1. A workflow for an algorithm designed to perform automated glycan detection in MALDI mass spectra using DHB/An and DHB/DMA matrices.

Formation of Schiff base in the DHB/An matrix, however, may be exploited for the purpose of automated identification of glycans if the mass spectra are acquired consecutively using both DHB/An and DHB/DMA. The workflow presented in Scheme 6.1 has been implemented to accomplish this task.

In the initial step, one is required to obtain mass spectra of the same sample in both matrices. A separate peak list is then generated for each spectrum. In the mass spectrum acquired in DHB/An matrix, peak pairs with a mass difference of 75 *m/z* units (ascribed to aniline Schiff base) are identified. Each peak pair tentatively corresponds to a native glycan (lower mass) and a Schiff base derivative peak (+75 *m/z*). In the next step, these pair selections are verified by searching for the lower mass peaks in the list generated for the sample run in DHB/DMA matrix. If peaks correspond, the following step is to confirm the absence of +75 *m/z* peaks in the DHB/DMA mass spectrum because there should be no aniline Schiff base derivatives formed in this matrix. This step also eliminates the occurrence of false positives resulting from unrelated peaks in the mass spectra, even if separated by 75 *m/z* units. If the cited criteria are met for a particular peak pair, the lower mass peak is then identified as a sodiated glycan, $[M+Na]^+$.

At first, this procedure was very useful for manual identification of oligosaccharides in the mass spectra, and the idea was further tested using a simple algorithm written as a Microsoft Excel-based macro (Microsoft, Redland, WA, USA). The mass spectra, processed using Bruker flexAnalysis software, were imported as Excel files before application of the algorithm. In Figure 6.2, all labeled glycan peaks (from chicken ovalbumin) were identified when the algorithm was applied to the corresponding mass spectral data.

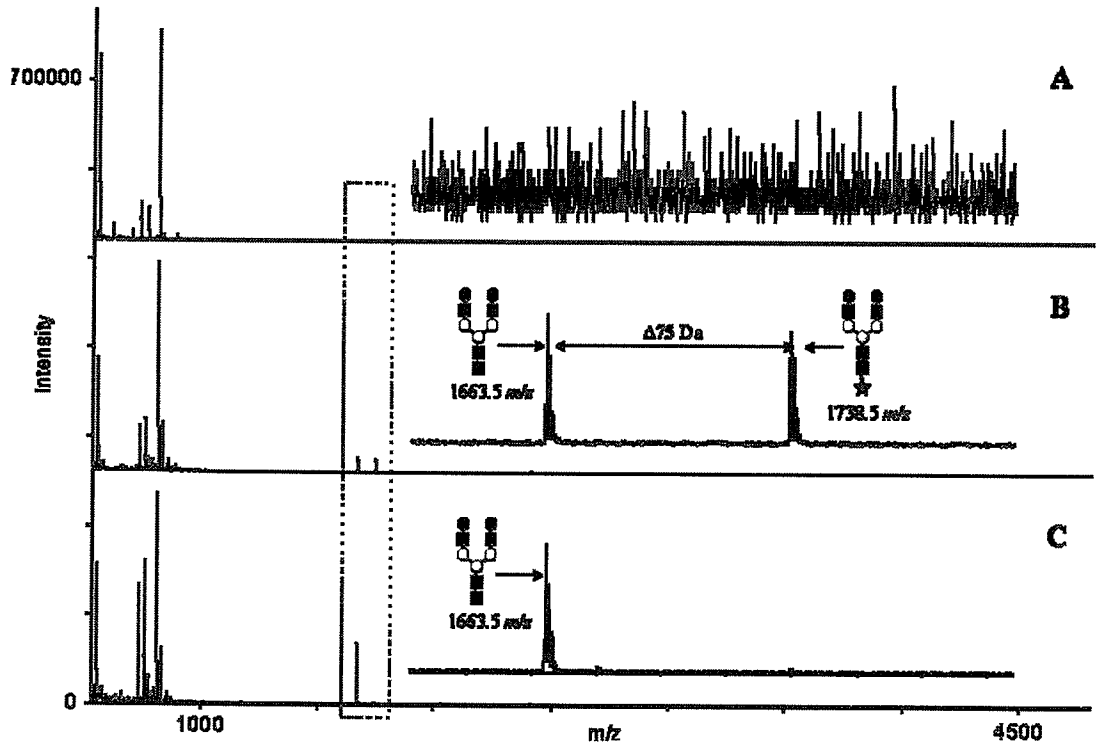


Figure 6.3. Reflecting mode MALDI-TOF mass spectra of α_1-acid glycoprotein peptide mixture containing glycopeptides treated with PNGase F acquired in DHB (A), DHB/An (B), and DHB/DMA (C). Expansions are shown in the insets. $[M+Na]^+$ ions of selected native glycan and its Schiff base derivative are labeled therein.

To conduct further identification tests, two additional sets of samples were prepared. The first sample consisted of an LC fraction of a tryptic digest of bovine α_1-acid glycoprotein containing a desialylated glycopeptide and several small peptides[18-20]. The sample was treated with PNGase F to detach the glycans from the glycopeptide. Mass spectra of this mixture in DHB, DHB/An, and DHB/DMA are given in Figure 6.3. The lower *m/z* region of the mass spectra displaying peptide $[M+Na]^+$ ions is identical for DHB/An and DHB/DMA matrices. Interpretation of this region is complicated by an extensive number of peptide sodium adducts, as observed in the previous reports on the

use of analogous matrix systems[18,19,23]. The two mass spectra clearly differ in the region displaying the $[M+Na]^+$ native glycan peak at 1663.5 *m/z* and the $[M+Na]^+$ aniline Schiff base peak at 1738.5 *m/z*, as seen in Figure 3. The algorithm identified only one peak as corresponding to a glycan, at 1663.5 *m/z*.

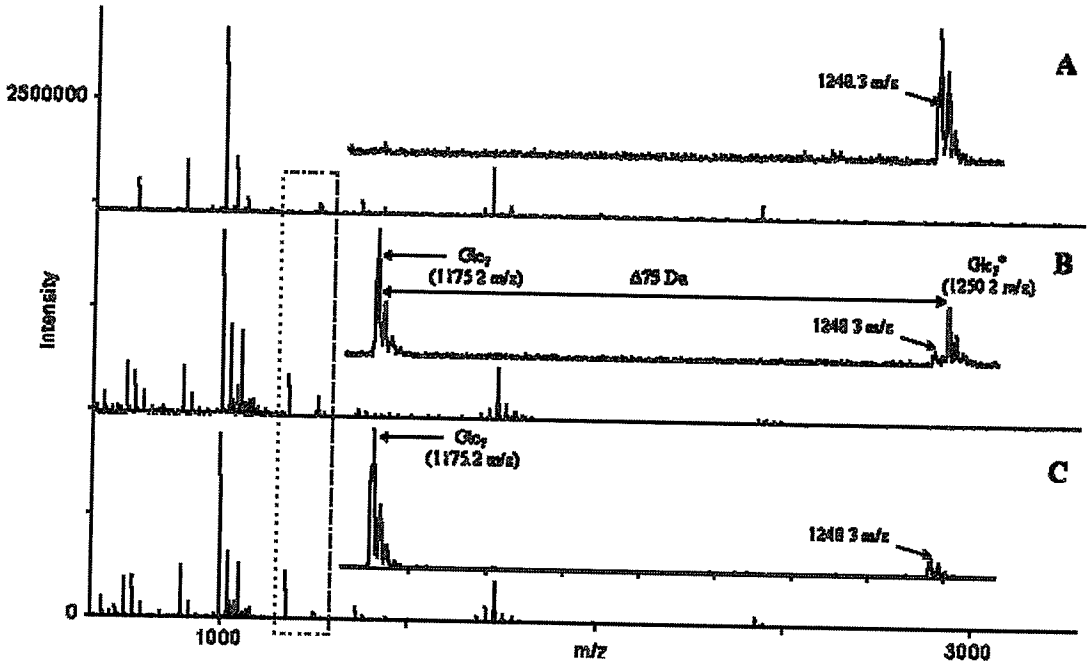


Figure 6.4. Reflecting MALDI-TOF mass spectra of a human α_1-acid glycoprotein tryptic digest mixture spiked with ~4 pmol of maltoheptaose acquired in DHB (A), DHB/An (B), and DHB/DMA (C). Expansions are shown in the insets. Note that the peak at 1248.3 *m/z* correspond to a $[M+H]^+$ peptide ion present in the mixture. Maltohepatose and is Schiff base derivative are observed as $[M+Na]^+$ ions.

The second sample tested was composed of a tryptic digest of human α_1-acid glycoprotein spiked with maltoheptaose. The mass spectra of this sample acquired in DHB, DHB/An, and DHB/DMA are given in Figure 6.4. The only difference in the mass spectra of the sample in DHB/An and DHB/DMA was due to the Schiff base derivative

of maltoheptaose formed in DHB/An. Despite the complexity of the mass spectra, maltoheptaose was identified by the algorithm as the only glycan present in the mixture (Figure 6.4 B).

6.5.3 Quantitative analysis of oligosaccharides: general observations

Previous work has shown that DHB/DMA matrix may be used for quantitative analysis of neutral oligosaccharides using MALDI-TOF MS[18,19]. This matrix system allowed for sensitive detection and accurate relative quantitation of glycans in a sample. Moreover, by using an internal standard, is was possible to estimate the absolute amounts of the analyte oligosaccharides deposited within a sample spot.

The main challenge in analyzing sialylated oligosaccharides samples by MALDI MS is associated with the labile and negatively charged nature of sialic acid residues. In-source and post-source losses of sialic acid often lead to inaccurate representation of the degree of sialylation of glycans[9]. Thus, sialylated glycans may be under-represented, whereas the relative amounts of the asialo species may be exaggerated in the mass spectra. Sialylated oligosaccharides give rise to $[M+(n+1)Na-nH]^+$ peaks in the positive ion mode and $(n+1)$ peaks are typically observed, where n is the number of sialic acid residues present in a given glycan. These peak patterns may serve as signatures for the presence of sialylated species in a sample. The occurrence of *N*-glycolylneuraminic acid (NeuGc) in glycans further complicates the mass spectra[20]. Chemical modification of sialic acid residues is often used to improve their stability for subsequent mass spectrometric analysis and to reduce the degree of sodiation, thus simplifying

interpretation of the mass spectra[24]. Negative ion extraction mode MALDI analysis has also been proven to be very useful in the analysis of sialylated oligosaccharides[25].

6.5.4 Analysis of sialylated oligosaccharides

The ionization patterns of sialylated *N*-linked glycans released from human α_1-acid glycoprotein were investigated in the positive ion mode, using DHB/DMA matrix in both linear and reflecting TOF modes, without chemical derivatization. Samples at three different levels of sialylation (untreated, partially desialylated, and fully desialylated) were prepared according to the procedure outlined in the Sample Preparation section.

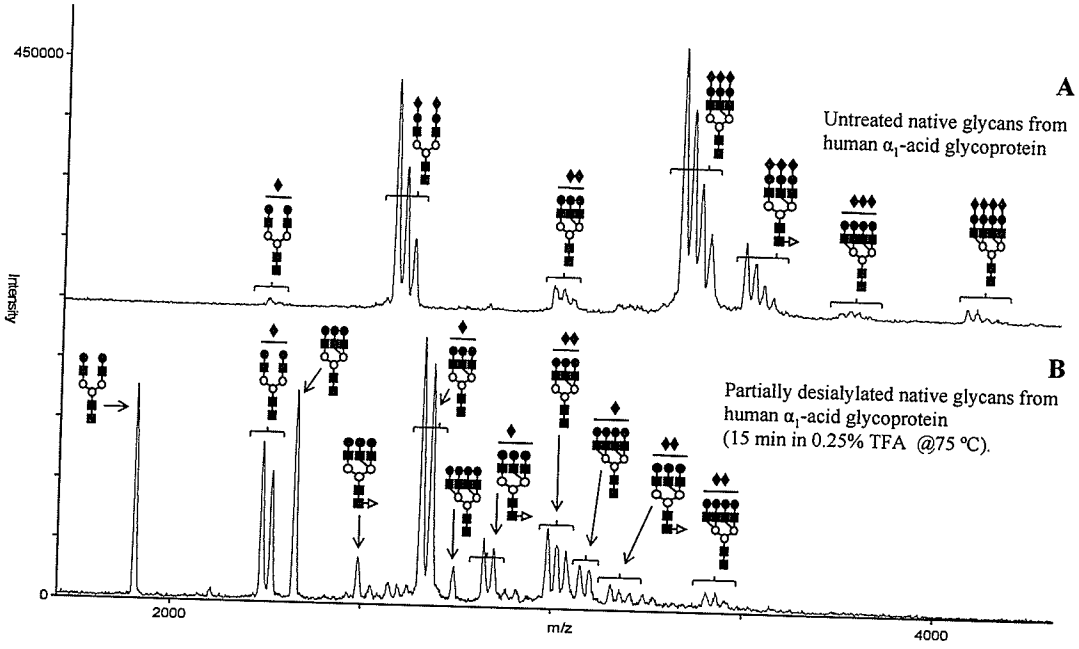


Figure 6.5. Linear mode MALDI-TOF mass spectra of the untreated sample (A) and partially desialylated sample (B) deposited in DHB/DMA matrix. The peaks corresponding to the $[M+Na]^+$ ions of some of the glycan structures observed are labeled in the mass spectra.

The degree of sialylation of the glycans was initially assessed in the linear mode using the untreated and partially desialylated samples (Figure 6.5). This was done primarily to evaluate the partial desialylation procedure employed in our method. It was important to have a sample with an intermediate degree of sialylation with respect to the untreated (fully sialylated) and fully desialylated sample. Mass spectra were acquired at the laser power setting yielding the highest abundance ratio of fully sialylated species to species one sialic acid short of full occupancy for the untreated and partially de-sialylated samples. This was done to minimize in-source desialylation. This setting (20% laser power) also resulted in the complete absence of fully de-sialylated glycan ions in the mass spectrum of the untreated sample (Figure 6.5 A), suggesting that all glycans in the sample have at least one sialic acid residue. Acquisition at a lower laser power resulted in a significant overall decrease of signal intensity, suggesting that the initial 20% setting was just above threshold for ionization of the glycans in the positive ion mode. Oligosaccharides with different degrees of sialylation were observed for both samples and various glycan structures were easily assigned to the peaks, as illustrated in Figure 6.5.

The same two samples, untreated and partially desialylated, were then analyzed in the positive ion mode using the reflector, at the same 20 % laser power setting as used earlier (spectra not shown). Surprisingly, no peaks corresponding to sialylated species were observed. Signal intensities of the asialo oligosaccharide ions remained relatively unchanged for the partially de-sialylated sample (within a 10% margin) with respect to those seen in the linear mode mass spectra. Also, the reflecting mode mass spectrum of the untreated sample lacked any peaks in the region of interest (i.e. above *m/z* 1500), in

sharp contrast with features observed in the linear mode mass spectrum (Figure 6.5 A). These observations imply that none of the sialylated glycan ions reached the second detector, likely due to post-source fragmentation. It is also clear that the laser power used was above threshold for the formation of oligosaccharide ions, as asialo glycan ions were abundant in the mass spectrum of the partially desialylated sample. Characteristically broad metastable peaks, often observed as a result of post-source fragmentation of analyte ions were completely absent from the mass spectra of both samples.

To further investigate these observations and to attempt quantitative analysis, all three samples were spiked with maltoheptaose as internal standard. The untreated sample contained maltoheptaose at approximately 15 pmol/μL (17 ng/μL), whereas the other two contained maltoheptaose at about 30 pmol/μL (34 ng/μL). The following volumes of spiked samples were deposited onto a MALDI target, as three spots: 2 μL of untreated sample and 1 μL of each of the partially de-sialylated and fully de-sialylated samples. Thus, twice the glycan material was deposited in the case of the fully sialylated sample relative to both others, while all three spots contained 30 pmol (34 ng) of maltoheptaose internal standard. The purpose of this experiment was to improve the detection of low abundance ions in the reflecting mode mass spectrum of the fully sialylated sample.

Mass spectra of these three samples, acquired in the reflecting mode, are presented in Figure 6.6. Amounts of the main four fully desialylated glycans in the three samples were determined relative to the maltoheptaose internal standard (by peak integration) and were all within less than 10% deviation for three successive acquisitions (see Figure 6.6). Sample composition, in terms of stable glycan structures without labile residues or groups and in terms of glycan antennarity, was thus obtained.

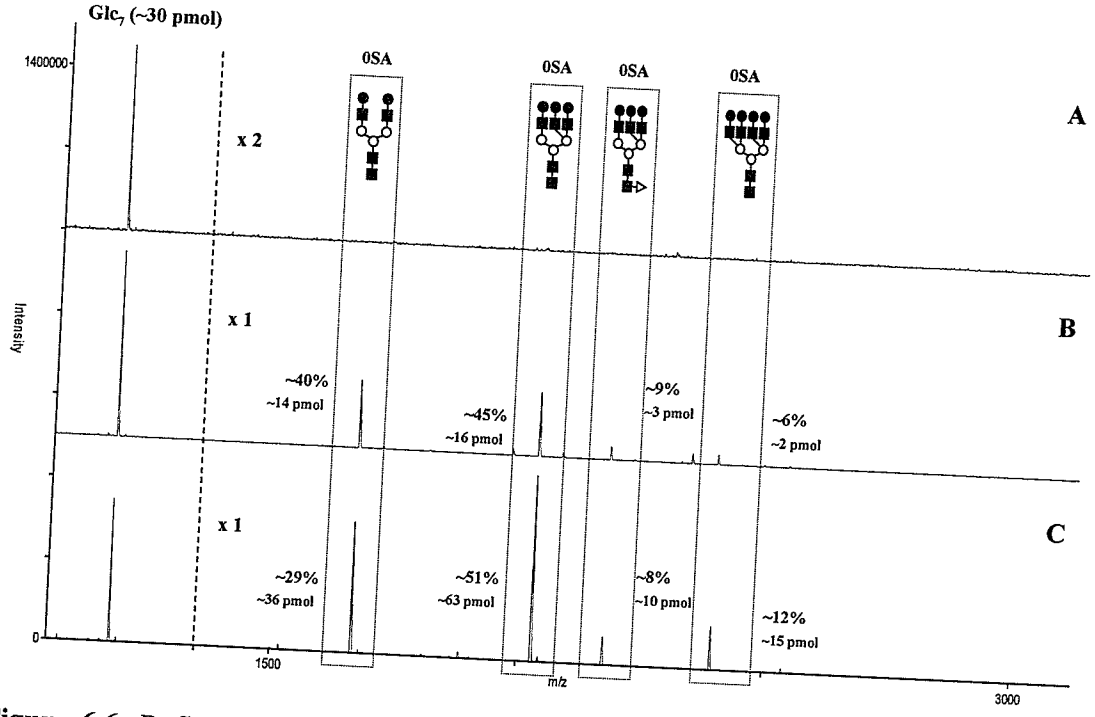


Figure 6.6. Reflecting mode MALDI-TOF mass spectra of the three glycan samples spiked with maltoheptaose in DHB/DMA matrix. (A) Untreated sample, (B) partially desialylated glycans, (B) fully desialylated glycans. The laser power used to acquire these mass spectra was identical to the one used in the acquisition of the mass spectra presented in Figure 6.5. All peaks correspond to $[M+Na]^+$ ions.

The same sample spots were then analyzed in linear mode. The mass spectrum of the fully desialylated sample contained only the $[M+Na]^+$ peaks corresponding to fully desialylated glycan structures. The relative abundances and calculated amounts of fully desialylated species in all three samples were identical to those calculated using the reflecting mode mass spectra.

When the laser power was increased from 20 % to 23 %, the relative intensities of the glycan signals in the mass spectra of the untreated and partially de-sialylated samples did decrease, as shown in Figure 6.7. Peaks corresponding to oligosaccharides with lower sialylation states increased in area, whereas those of glycans with a higher number of

sialic acid residues decreased with respect to areas observed in Figure 6.5. In the reflecting mode (not shown), $[M+Na]^+$ signal intensities of asialo glycans appeared to increase with respect to those observed in Figure 6.6 (using the optimal (20%) laser power). Interestingly, peaks corresponding to metastable ions of the sialylated species together with peaks corresponding to sialylated species began to emerge at this laser power (23%), although only at low signal intensity.

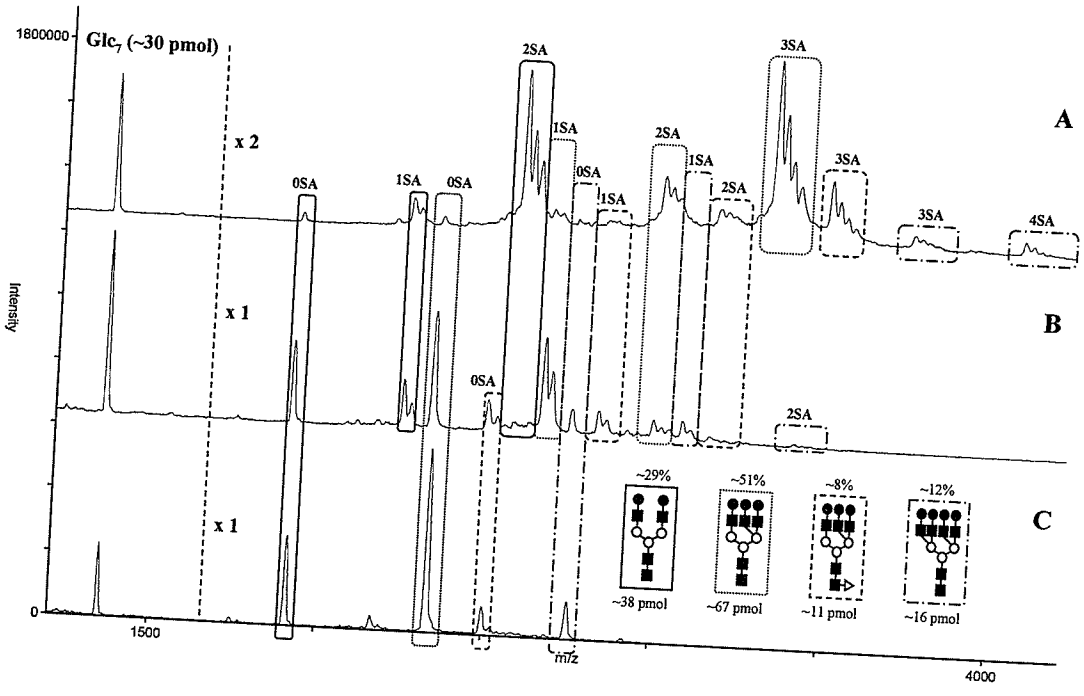


Figure 6.7. Linear mode MALDI-TOF mass spectra of oligosaccharides detached from α_1-acid glycoprotein in DHB/DMA matrix acquired at a slightly higher laser power. (A) Untreated sample, (B) partially desialylated glycans, (B) fully desialylated glycans. Peaks corresponding to $[M+Na]^+$ ions of the four dominant glycan structures with varying degree of sialylation are labeled. Note the spectral differences relative to the mass spectra shown in Figure 6.5.

On the basis of these observations on the analysis of sialylated oligosaccharides in the linear and reflecting modes, it is possible to propose a method for relative and absolute quantitation of oligosaccharides by positive-ion MALDI-MS.

In the first step, the laser power and sample size are optimized to produce a strong signal in the linear mode without any noticeable in-source loss of sialic acid, using an untreated sample. At this optimal laser setting, there should be no signal observed for sialylated glycans in the reflecting mode. Asialo glycans are then quantified using an appropriate internal standard, preferably in the reflecting mode[18,19] There would be no interference from sialylated glycan peaks.

In the second step, a portion of the fully desialylated sample is analyzed under the same conditions as cited earlier, in both linear and reflecting modes, and asialo glycans are again quantified. For each glycan type, the difference between the calculated amount of the asialo glycans in untreated and fully desialylated samples corresponds to the total amount of sialylated species. Thus, (i) determining the abundance ratios of glycans of a particular type at different levels of sialylation in the linear mode in the untreated sample and (ii) applying these ratios to the difference calculated above, make it possible to indirectly determine the amount of each sialylated species for each glycan type present in the untreated sample.

This method does not involve the use of sialic acid-containing oligosaccharide standards. Also, neutral glycans may be employed as internal standards for the analysis of acidic glycans[18,19]. This method rests on the assumptions that no in-source losses of sialic acid occur under the chosen conditions, and that the ionization efficiency of sialylated glycans does not depend on the number of sialic acid residues. These assumptions hold

for the purpose of estimating – with reasonable precision and accuracy – the quantities of sialylated oligosaccharides contained in a sample.

6.6 Conclusions

This study demonstrates applications of DHB/An and DHB/DMA matrices for MALDI-based mass spectrometric analyses of oligosaccharides from glycoproteins. The use of both matrices allows for simple automated identification of glycans because of their partial derivatization with aniline and subsequent application of an algorithm-based mass difference discrimination procedure. Thus, the occurrence of both the aniline Schiff base and native glycan signals may be exploited as a key variable in data processing for automated detection and identification of glycans. The use of DHB/DMA matrix for quantitative analysis of native neutral oligosaccharides presents advantages over other MALDI-based quantitative analysis methods in terms of simplicity and sensitivity. It was shown how sialylated glycans may be analyzed quantitatively using DHB/DMA MALDI matrix. The algorithm discussed herein may also be used for automated identification of sialylated glycans, although additional adjustments to the peak identification procedure would have to be made to account for the multiple peaks representing all sialylated species in a sample.

6.7 Acknowledgements

The authors thank The Natural Sciences and Engineering Council of Canada (NSERC), the Canada Research Chairs program (CRC) and the Canadian Foundation for Innovation (CFI) for funding. The authors also gratefully acknowledge Colin Lee and Dr. Vincent C. Chen for their invaluable technical assistance and helpful discussions.

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Chapter 7:

Conclusions and future work.

7.1.1 MALDI matrices for analysis of oligosaccharides

The use of several amine compounds, specifically aniline and *N,N*-dimethylaniline, together with DHB as a MALDI matrix was found to be particularly useful for the analysis of native oligosaccharides in positive mode MALDI MS, as outlined in Chapters 4-6. Many examples pertaining to applications of similar binary MALDI matrices, collectively termed "ionic matrices", are found throughout the recent literature in the context of peptide analysis[1]. However, investigation of their use for the analysis of sugars has been somewhat limited. The work presented in this dissertation attempted to fill this void by providing several convincing examples of the practical benefits of using such materials for the analysis of carbohydrates.

The key "selling point" of these new techniques is their ability to generate a very uniform matrix crystal layer upon deposition of the sample onto a MALDI target, which consistently results in very homogeneous sample distribution over the sample spot. This ultimately leads to improved sensitivity and enables quantitative analysis of oligosaccharides by MALDI MS. However, these methods are not "uniquely fitted" to produce these results. A very careful manipulation of factors such as matrix solution concentration, solvents used, target surface properties, and sample-drying temperatures has a profound effect on the sample crystal morphology. In subsequent unpublished work, it was shown that similar crystal morphologies could be attained without using of any additives, leading to similar enhancements in spectral quality (results not shown). On the basis of these observations, one is inclined to conclude that the physical properties of the sample crystal are mainly responsible for the reported improvements in the analysis of neutral native sugars, and that the chemical nature of the additive, essentially minor

effect. While true to a large extent, this observation does not negate our work, as the additive does conveniently facilitate the formation of crystals with favorable physical properties under common laboratory conditions.

It would be interesting to extend this project to the analysis of acidic glycans. Work presented in Chapter 6 demonstrates a significant improvement in the ionization of acidic sialylated glycans with DHB/DMA matrix in positive ion mode. This has been confirmed independently by a group of collaborators, who are studying the ionization of acidic sulfated glycans (Snovida *et al.*, personal communication, 2009). Thus, the chemical properties of the additive, particularly ion-pairing with DHB, may play a key role in these phenomena, and this would require further investigation. Systematic analysis of several different types of acidic glycans in both DHB and DHB/DMA (or similar ionic matrices), as well as spectroscopic studies of these matrices, may provide answers to some of the fundamental questions pertaining to MALDI mechanisms.

7.1.2 Isolation of sialoglycopeptides by capillary electrophoresis

The use of CE for isolation of acidic sialylated glycopeptides was demonstrated as a "proof of concept" in the work described in Chapter 2. Although the method did achieve a reasonable level of desired separation, it was developed and validated using only a single standard glycoprotein. This does not address the full scope of glycoproteomics, as the method may not be very effective for samples having a large proportion of non-glycosylated acidic peptides, i.e. peptides with a high number of acidic amino acids or acidic modifications, such as phosphate groups. Since the overall pI of a molecule is based on the pKa values of its individual ionizable groups, these acidic

peptides are likely to co-elute with sialylated glycopeptides. Likewise, sialylated glycopeptides which bear a large number basic amino acids may not be isolated into the "acidic" fractions at all. Thus, attempts at targeted isolation of acidic glycopeptides by this method may not lead to the desired result, especially if the samples consist of a large number of proteins at different levels of expression.

In addition to the problems outlined above, general method development and transfer procedures associated with capillary electrophoresis are very difficult, relative to those of chromatographic methods, due to exceptional responsiveness of CE to impurities in both sample and buffer solutions. Direct method transfers to different samples may therefore not always be possible.

Finally, very low sample load capacity limits capillary electrophoresis as an adequate separation technique for preparatory work in general. While making it a very powerful technique in terms of sensitivity and resolution for routine dedicated analyses, it leaves CE vastly inferior relative to LC in terms of scalability for sample purification purposes.

I believe that a chromatographic approach would be more practical and efficient for the purpose of glycoproteomic/glycomic studies, and that comparable separations may be achieved without invoking the use of CE. However, as outlined in the next section, the use of CE should be explored further at the glycomic level, particularly for experiments involving the isolation of acidic glycans and for resolution of structural isomers.

7.2 Future work

7.2.1 General perspective

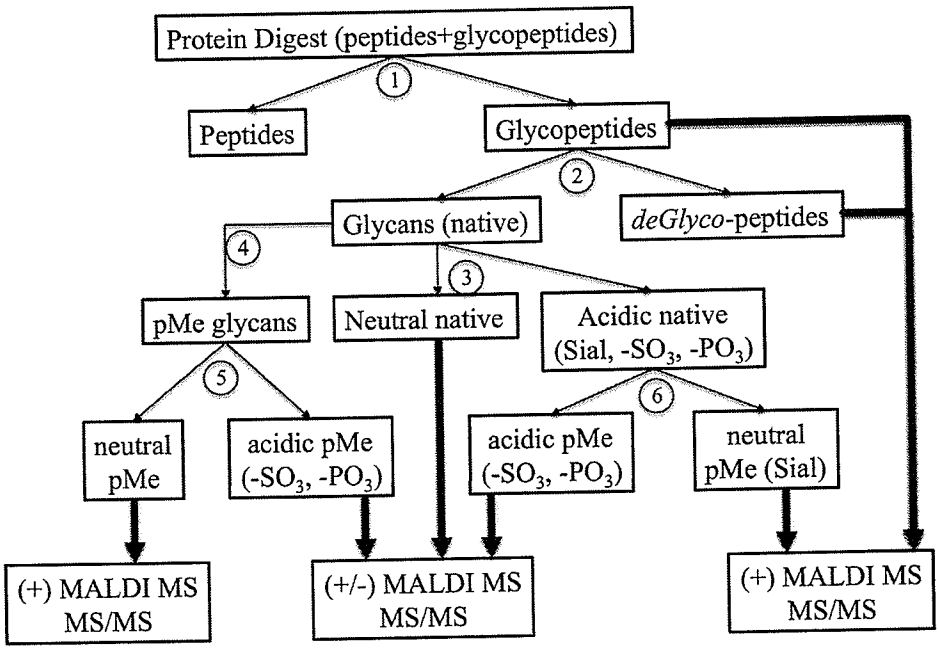
There still remain many limitations pertaining to the currently available methods for MS-based glycoproteomic and glycomic analyses. The main challenges related to structural analysis of oligosaccharides stem from poor ability to resolve structural isomers (physically, with chromatographic and electrophoretic techniques and, often, spectrometrically, at the tandem MS level). Also, the analysis of oligosaccharides bearing acidic modifications, such as sialic acids and sulfate groups, is complicated, as it may involve switching between positive and negative ion modes in order to improve detection sensitivity of these negatively-charged glycans and often requires specialized sample preparation protocols due to their labile nature. Finally, there is a constant demand for improvements of analytical sensitivity in general, which is critical especially for studying glycans occurring in low abundance. Hence, more method development work needs to be done and tested in these areas in order to fully exploit the practical advantages and theoretical potentials of MS-based technologies in the field of glycomics. Specific aims of future work should incorporate the following elements according to the workflow presented in Scheme 7.1:

(i) Improving the methods for isolation of glycopeptides from complex protein digest samples for subsequent analysis by mass spectrometry (MS). The work would include further development of glycomic workflows at the glycopeptide level.

(ii) Further development and optimization of chromatographic and electrophoretic procedures for targeted fractionation of glycans occurring within a diverse mixture on a basis of presence of some key structural/chemical elements, such as sialic acids and sulfate groups.

(iii) Application of these methodologies to analysis of sulfo-glycomes of various tissues and organisms – a niche in biology, which is receiving increasingly more attention, mainly due to newfound recognition of the significance of sulfate-containing epitopes (in both *N*- and *O*-linked glycans), as well as the challenges associated with these studies. This is to be done with a heavy emphasis on the use of state-of-the-art MS-based techniques.

All numbered steps in Scheme 7.1 require further development. Once optimized and validated, they ought to be applied to studies of real samples for complete characterization of their glycomes, particularly sulfo-glycomes.



Scheme 7.1. The overall proposed workflow designed to attain stated research objectives, i.e. improving glycomic analysis at the glycopeptide level and fractionating glycans according to occurrence of sialic acid, sulfate/phosphate groups.

7.2.2 Glycopeptide enrichment procedures

Glycomic studies at the glycopeptide level are not only aimed at identification of glycosylation sites in a given glycoprotein, but also at a complete analysis of the microheterogeneity of glycoforms found within a single site – information offered exclusively at this level[2]. In order to establish an efficient workflow for the analysis of glycopeptides, there must be a method available for purification/enrichment of glycopeptides which is both facile and high yielding. Currently used glycopeptide purification methods are usually limited by low recovery levels and incomplete separation of glycopeptides, which are often only present in trace levels, from concomitant peptides in complex proteolytic mixtures[3,4].

Recent work on the development of more efficient liquid chromatography (LC) based techniques offers potential for addressing these issues and promises both high enrichment yields and lower peptide carryover rates. There is much more work to be done in this area of research. More specifically, the work should involve experimentation with several chromatographic phases (natural carbohydrate polymers, lectins, synthetic materials) to test their targeted retention capacity and to compare their efficiencies. Also, construction of chromatographic columns and development of solvent systems that would facilitate quick and efficient workflows should be compared using these different packings. Full validation of these protocols should be carried out on real-life complex samples. MS analysis, with a heavy emphasis on both on- and off-line LC separations, would be an integral part of this work.

Upon isolation of the glycopeptide material, global glycan profiling may be performed, all glycoproteins present in the sample may be identified (through database

peptide MS/MS searches for deglycosylated peptides), and, alternatively or in parallel, reversed-phase chromatography may be performed on a sample consisting of isolated glycopeptides with a small spiked portion of the same deglycosylated peptides. This step, in theory, would allow for isolation of all glycopeptides with a common peptide chain, which differ only by the attached glycoforms, provided that a good separation on the basis of differences in the peptide part is attainable. In a fashion similar to some currently popular sequence specific peptide retention prediction algorithms[5] (designed for use in proteomics), one may predict an elution window of all glycopeptides (with relatively poor ionization efficiency relative to their common peptide) related through their peptide chain on the basis of elution time of the deglycosylated peptide. As a spin-off from this work, it would also be interesting to study the effects of various glycoforms on retention of the glycopeptides. Once isolated, site-specific analysis may be carried out.

7.2.3 Sulfo-glycomics

Glycomic work at the glycan level often requires fractionation of samples prior to MS profiling in order to group components on the basis of size or charge and to improve analytical sensitivity towards the low abundance glycoforms (by minimizing ionization suppression by the more abundant species). These steps aid in providing better qualitative and quantitative information about the samples. Furthermore, the workflows associated with these procedures facilitate studies of specific classes of glycans (sialylated and sulfated, for example) separately, and, in conjunction with various derivatization methods, would allow for more in-depth structural characterization of various glycoform types found within a wide range of glycan samples. Particularly in the cases of glycans

with sialic acid, sulfate, or phosphate containing epitopes – functional groups which are responsible for many biologically crucial events, such as mannose-6-phosphate, sialyl Lewis, or sulfo-sialyl Lewis epitopes in specific antigen/receptor recognition events, oncological metastases, and others, but which are also often difficult to detect and characterize with sufficient accuracy mainly due to low abundance and their labile, negatively-charged nature[6,7].

Sulfo-glycomics is an emerging new frontier in the field of glycomics, as increasing numbers of sulfate-containing glycan epitopes are found to play important roles in biology[8]. Few studies aimed at characterization of sulfate-containing epitopes in glycoproteins have been carried out to date, mainly due to the lack of sufficiently sensitive techniques and efficient workflows. However, recent introduction of the more accessible and sensitive, functionally wide-ranging MS-based instruments (OrbiTrap™ based instruments and electron-transfer dissociation fragmentation technology)[9,10] promises to be of great value in enabling the study of these biomolecules. Together with ongoing developments in chromatography-based purification techniques aimed at recovering the less abundant and often undetected sulfo-glycoforms, these technologies will deliver new insights into the mechanisms of many intricate biological processes. Capillary electrophoresis techniques may also prove to be a useful tool in addressing problems of separation and detailed characterization of structural isomers of important glycoforms[11].

I have been involved in the development of such separation methods and would like to broaden this research. It is possible to completely separate and identify neutral glycans, sialylated acidic glycans and sulfated/phosphorylated glycans by sequential

chromatographic fractionations (using materials such as a weak anion exchanger aminopropyl phase, for instance), permethylation steps followed by MS analysis[12-14]. However, more work is required for optimization of these procedures and extensions of the methods in order to include other, more exotic, types of glycans/glycoconjugates. Experimentation with a diverse range of classical and novel types of stationary/extraction phases (capable of weak anion exchange) and solvent systems, capillary elecrophoresis, as well as MS-based validation, would lead towards this goal. Furthermore, experiments involving ion mobility mass spectrometry may be helpful in establishing on-line non-chromatographic methods for separation of structural isomers of glycans[15].

Identification and characterization of sulfated epitopes will be a challenging task, mainly because of their low abundance, their negative charge, and labile nature. I would like to extend currently available, as well as those still under development, techniques and workflows to studies of glycoproteomes of various tissues, particularly as it relates to abundance levels and detailed structural studies of biologically important sulfated glycoforms.

7.3 Closing summary

I would like to stress that particular emphasis of future work should be on improving yields (nearly complete recovery/isolation rates) of glycopeptides and targeted glycans in a facile and economically efficient manner, which would facilitate quick and detailed qualitative/quantitative analysis of glycoproteomes/glycomes and which would be suitable for automated workflows. Inexpensive materials such as cellulose and Sepharose have thus far been identified as the best candidates as stationary extraction

phases appropriate for efficient extraction of glycopeptides in the preliminary experiments. Aminopropyl phase also has a high potential to work reliably well for the purpose of separating neutral and acidic glycans, in both native and permethylated forms. All steps require minimal handling and clean-up – a significant improvement over some currently employed methods. I am confident that the outlined aims will materialize and that they will be of benefit to the scientific community in this field. I look forward with much excitement towards further development of this work and to the next chapters of my scientific career.

7.4 References

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