THE ANALYSIS OF GLYCOPEPTIDE OLIGOSACCHARIDES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY – CHARGED AEROSOL DETECTION

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Abstract

of

THE ANALYSIS OF GLYCOPROTEIN OLIGOSACCHARIDES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY – CHARGED AEROSOL DETECTION

by

Katherine Hu

Glycoproteins have been a topic of interest for many years because of the important roles they play in many diverse biological functions such as molecular recognition. For many glycoproteins, the key to their functions are their oligosaccharide moieties. However, existing methods of analysis are often labor intensive or costly. Previously, my research group developed a method using high performance liquid chromatography with charged aerosol detection (HPLC-CAD) that was sensitive without requiring derivatization or exact standards. The earlier work used an amino column, but the method was limited in sensitivity due to column bleed and gave moderate resolution. In this presentation, I discuss the use of a porous graphitic carbon (PGC) column. A 3 µm particle diameter, 100 x 4 mm Hypercarb PGC column was used for these studies. Both reducing oligosaccharides and non-reducing oligosaccharides were investigated for use as calibration standards. The reducing oligosaccharides gave multiple peaks due to separation of alpha and beta anomers, making calibration difficult. Solutions of reduced oligosaccharide standards (linear chains and cyclodextrins) were made at different
concentrations and analyzed by HPLC-CAD with the PGC column using gradient elution. The ion voltage of the home-built CAD instrument was optimized and calibration curves produced. Sugars reduced by sodium borohydride were able to test the methodology over a greater range of possible oligosaccharides and to match the reaction used to cleave oligosaccharides from glycoproteins. The reduction of oligosaccharides was a success, with near 100% reduction of maltotetraose to maltotetraitol using sodium borohydride and generally with good precision. The analysis of oligosaccharide standards on the PGC column showed excellent linearity with optimized ion voltages. The detection limit was found to be 0.2 ng, while previous results with the amino column had detection limits near 1 ng. The current method also resulted in two times the peak capacity due to higher separation efficiency. The PGC column has demonstrated improvements in the analysis of oligosaccharides by providing increased sensitivity and linearity over the amino column. Glycoproteins such as immunoglobulin G and ribonuclease B were used to test the method for analysis. Our method showed good chromatographic results. With further work, complete resolution of peaks may be obtained.

________________________, Committee Chair
Dr. Roy Dixon

________________________
Date
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BACKGROUND

Importance of Glycoproteins

Glycoproteins have been a topic of interest for many years because of the important roles they play in many diverse biological functions such as molecular recognition, development of the embryo, immune defense, inflammation, viral replication, and fertilization. Glycosylation is a critical post-translational process since many proteins in cells and biological fluids are glycosylated. It is estimated that over 50% of all mammalian proteins are glycosylated [1]. Furthermore, glycoproteins are present in animals, plants, microorganisms, and viruses, so it is not surprising that a lot of effort has been put forth to study glycoproteins [2].

For many glycoproteins, the key to their function is their oligosaccharide moieties. For example, cancer cells that can metastasize often have sialic acid-rich glycoproteins due to the activation of glycosyl transferases and abnormal glycosylation in cancer cell membranes. The sialic acid moieties increase the cell membranes’ capability to stick to vascular endothelium and decrease the ability of cancer cells to be destroyed by host defense mechanisms. These sialic acid moieties also play a role in the transport of positively charged compounds, cell-to-cell repulsion, and masking antigenic determinants on the receptor molecules [3]. Another example is the P-glycoprotein (Pgp), which is frequently over-expressed in drug-resistant cancer cells. Pgp, encoded by the multidrug resistance 1 (MDR1) gene, operates as a pump to eliminate anticancer drugs from cancer cells and regulates the distribution and bioavailability of drugs. An increase in Pgp
expression can reduce the absorption of drugs, but an increase in drug toxicity can result when there is a decrease in Pgp expression. Therefore, it is crucial in pharmacokinetics to develop a drug that can target Pgp for chemotherapy treatment [4].

Another example of the biological importance of the oligosaccharide moieties conjugated to glycoproteins can be found at fertilization. Prior to ovulation, mammalian oocytes secrete glycoproteins to form an extracellular coat termed the zona pellucida (ZP) which is a three-dimensional network of cross-linked filaments that interacts with sperm to regulate fertilization. The oligosaccharide moieties on the ZP glycoproteins not only serve as receptors for species-specific recognition of sperm prior to fusion, but they also play a role in preventing polyspermy (multiple sperm entry into an egg) after a single sperm has fused [5]. With such a broad range of glycoprotein functions, investigative studies are prevalent in this field.

**Structure of Glycoproteins**

A glycoprotein, shown in Figure 1, consists of a protein which is glycosylated with oligosaccharides covalently connected through the nitrogen atom of an asparagine (N-linked) or an oxygen atom of a serine or threonine (O-linked). The carbohydrate moiety, also known as the glycan, can play a role in direct recognition functions, but can also alter the properties of the protein. The combination of possible monosaccharide attachments sites along with the moderate number of different monosaccharides can lead to a very large number of possible glycan structures. Oligosaccharides can be large in size which may allow them to cover active sites of proteins, to modulate the interactions
between other molecules, and to affect the rate of conformational changes. Even small oligosaccharides have been known to shield large areas of a protein surface [6].

Post-translational glycosylation, which is the linking of saccharides to proteins, allows for a wide variety of structures which can therefore perform different functions. There are two main types of glycosylation: N-glycosylation (N-linked), where the oligosaccharide is β-glycosidically attached via N-acetylglucosamine (GlcNAc) to the amide group of asparagines with an Asn-X-Ser/Thr motif and O-glycosylation (O-linked), where the oligosaccharide is α-glycosidically attached via GlcNAc and GalNAc (N-acetylgalactosamine) to hydroxyl groups on Ser or Thr [1]. Both types of glycans are widespread on the protein where they aid in cell-to-cell communication [7].

Figure 1. An illustration of a typical glycoprotein. From http://www.utm.utoronto.ca/~w3bio315/lecture2.htm.
Monosaccharides are the most basic units of carbohydrates and the building blocks of nucleic acids. They usually contain three to nine carbon atoms, which contribute to size stereochemistry variation. Examples of monosaccharides are shown in Figure 2 in their Fischer projections. There are seven monosaccharides that are prevalent in glycans attached to proteins in humans: glucose, mannose, galactose, N-acetyl-glucosamine, N-acetyl-galactosamine, fucose, and sialic acid [8]. Oligosaccharides are formed when two or more monosaccharides are linked by glycosidic bonds and can be in charged or uncharged form. Charged oligosaccharides will have sialic acid monosaccharides. O-glycosidic bonding occurs when the anomeric carbon atom bonds with the hydroxyl oxygen atom. N-glycosidic bonds can occur when the anomeric carbon atom links to the nitrogen atom of an amine. The monosaccharides can be linked in linear or branched structures in a variety of sequences and lengths, resulting in a diversity of structures. Monosaccharide-monosaccharide bonding can occur through a variety of connections, such as C4→C1, C6→C1 [6].

![Monosaccharide structures](image)

**Figure 2.** Fischer projections of monosaccharides. Glucose, galactose, and mannose are shown.
The synthesis of glycans is not template driven and does not have a proofreading process, which results in a diversity of oligosaccharide structures. The differing glycan chains provide glycoproteins with different conformations, resulting in their ability to perform different tasks. The stability, protease resistance, or quaternary structure of proteins can be altered by the attached oligosaccharides. Consequently, researchers have concluded that there is no single unifying function for oligosaccharides. However, with a non-templated process, glycosylation is highly susceptible to changes in cellular function, and abnormal glycosylation may result in a large number of diseases, including cancer. Therefore, a variety of possible glycans can possibly attach to a specific amino acid site on a protein [9].

In pharmaceuticals development, the ability to make an effective protein drug can be dependent on proper glycosylation of the protein. Having specific glycosylation patterns can highly affect a drug’s bioactivity, half-life, and immunogenicity due to its influences on the functional characteristics of a protein. The amino acid structure of the original product can be reproduced, but a given glycosylation pattern is much more difficult to achieve [10, 11]. For this reason, being able to profile and quantify all of the oligosaccharides on a glycoprotein may be able to help us further comprehend the complex structure of glycans and their role in biological functions.

**Past Work on Obtaining Glycan Profiles**

There are a few different elements associated with glycoprotein analysis. Before glycans can be studied, the glycoprotein itself needs to be isolated in significant amounts.
First, cells or tissue containing the glycoprotein of interest will need to be obtained and will likely need to be homogenized for further separation. The glycoprotein can then be purified using chromatographic methods such as gel filtration, ion exchange, and affinity chromatography. Gel filtration separates the smaller proteins from larger ones by using porous beads or packing material made from polyacrylamide or agarose. Ion exchange separates proteins based on their charge by using beads with carboxylate groups. Using affinity chromatography, proteins will bind to beads with specific ligands based on binding affinity. The glycoproteins can then be eluted from the column with a solution that decreases the binding affinity of the protein to the ligand [6].

In addition to gel filtration, ion exchange, and affinity chromatography, gel electrophoresis can also be used and is often very efficient. However, traditional SDS-PAGE techniques often result in broad bands due to a heterogeneous glycosylation pattern due to the non-templated glycosylation process. Utilizing 2D gel electrophoresis often resolves this problem by efficiently separating the various glycoforms but frequently under-represents membrane glycoproteins due to the low solubilizing power of the detergents used. The use of SDS-PAGE in conjunction with HPLC after the solubilization of the proteins with detergents and chaotropic agents has also been used with better results [1].

After glycoprotein purification, the glycan can then be profiled. With regards to the site-specific glycosylation properties, a glycopeptide analysis must be completed by digestion with endoproteinases. This results in a mixture of peptides and glycopeptides.
Enrichment techniques such as lectin affinity and sequential lectin chromatography can be employed to separate glycosylated from non-glycosylated glycopeptides. Alternatively, glycopeptide mixtures can be fractionated by HPLC with ESI-MS for selective detection of glycopeptides [1].

Then the glycans can be released from the protein or peptide backbone by enzymatic or chemical methods. The enzymatic release method produces most of the intact oligosaccharides and peptides or proteins, but not all. The most common enzyme used for release is the PNGaseF amidase. It releases many N-linked glycans except those with a α1-3 linked fucose attached to the reducing end of GlcNAc. For example, it can cleave the linkage between GlcNAc and asparagine, while converting the asparagine to aspartic acid. Unfortunately, there is no such enzyme that will universally cleave O-linked glycans at their amino acid attachment site. Alternatively, the chemical release methods destroy the non-carbohydrate substituents, so any information about the glycosylation site is lost [1]. The most common process for chemical release is hydrazinolysis, where anhydrous hydrazine is added to the glycoprotein sample.

Unreduced O- and N-linked oligosaccharides are completely released whereas the protein is destroyed. With β-elimination methods, sodium hydroxide is used to cleave the glycan from the peptide. Then, sodium borohydride reduces the glycan at the anomeric carbon.

While the determination of the glycoprotein structure or even glycan structure will require many more analysis steps, glycan separation and detection by itself is useful in characterizing glycosylation patterns. For example, cancerous cells have different
glycosylation patterns when compared to normal cells, and genetically modified proteins often have different glycosylation patterns as compared to their native counterparts. Additionally, glycan separation is often combined with mass spectrometric methods to determine glycan structure. My thesis will be focused on developing methodology to profile glycans, which may be used in conjunction with other glycoprotein information to advance our knowledge of their structure and/or function or also for purposes of comparing related glycoproteins.

1) Analysis of Underivatized Sugars

One of the most common methods to detect released glycans involves fluorescence labeling and then structure analysis [12]. Existing methods of analysis are often labor intensive, costly, or both, as is discussed in more detail in the following section. Several detection methods without derivatization are available, but these have significant limitations. High performance liquid chromatography (HPLC) is usually used to separate oligosaccharides, but direct UV detection is only sensitive enough to detect weakly absorbing sugars at moderate to high concentrations [1]. Also, detection depends on the proportion of amide containing monosaccharides (sialic acid, N-acetyl-glucosamine and N-acetyl-galactosamine) since these are the UV absorbing constituents, which varies from glycan to glycan [13]. High-performance/high-pH anion exchange chromatography (HPAEC) coupled with a pulsed amperometric detection (PAD) is also a technique used [1]. It is fast, efficient, and sensitive for separating and detecting oligosaccharides based on size, structure, linkage and branching, and does not require labeling. However, HPAEC-PAD requires a high salt content, which makes it difficult to
isolate oligosaccharides for further tests, such as MS analysis. Also, the detector response can depend on the structure of the oligosaccharide [1]. In addition, quantitation without standards is problematic, since glycan response is variable. The peak areas do not reflect concentration accurately [10]. Another drawback with analyses at high pH is that there is an increased epimerization rate of GlcNAc to ManNAc [1].

Mass spectrometry (MS) has been the leading technique for characterizing glycans because of its sensitivity. Also, structural information can be obtained through 2-dimensional mass spectrometry techniques. For example, O-linked oligosaccharides chemically released from *Xenopus laevis* egg jelly was analyzed by the use of collision-induced dissociation MS to produce fragment ions that can be structurally identified from a catalog library of oligosaccharide structures [14]. In addition, a series of exoglycosidase digestions were used in combination with MALDI Fourier transform ion cyclotron resonance (FTICR) MS to structurally determine the glycan structures [14]. A comparison study showed that LC-MS without derivatization can give reasonable results, although MS analysis following derivatization is more common [12].

2) **Analysis of Derivatized Sugars**

Due to the lack of chromophores found in oligosaccharides and their low ionization efficiency for MS analysis, chemical derivatization is often used to resolve these issues. In particular, reductive amination, chromogenic or fluorescent tags can be added to the glycan to significantly increase the sensitivity of detection. Figure 3 shows examples of different derivatization processes, which are described below.
Figure 3. Glycan derivatization methods. 
A) 2-Aminobenzoic acid; B) permethylation.

Permethylation derivatization can convert all hydroxyl groups to methoxyl groups and stabilize the sialic acid residues by converting them to methyl esters. This improves compound behavior in MALDI-MS [15]. Although permethylated glycans can be readily separated using conventional reversed phase chromatography, spectroscopic detection is still difficult without adding chromogenic or fluorescent tags. Another common method is utilizing pyridilamine (PA) for fluorescence tagging followed by HPLC with fluorescence detection. Drawbacks of using PA include: complicated multistep reactions, the need to remove unreacted PA before HPLC analysis, and loss of N-linked sugar chains [15]. Anthranilic acid (AA) is one of the smallest fluorescent labels for carbohydrate analysis, and is therefore preferred over other labels. It is highly fluorescent,
resulting in very high sensitivity. It is also specific for labeling reducing monosaccharides and oligosaccharides, and is charged, allowing analysis by CE and improved ionization efficiency using LC-MS. Another fluorescent label, aminobenzamide (AB), is commonly used with mass spectrometry. However, the sugars need to be re-N-acetylated before derivatization can occur, making it less appealing [10].

Another method for analyzing oligosaccharides is capillary electrophoresis (CE), which separates charged analytes based on migration velocity in an electric field. CE provides high separation efficiency and speed. The migration velocity of each oligosaccharide is determined by its electric charge/molecular size ratio. However, neutral oligosaccharides must be converted to charged species or tagged with AA (anthranilic acid) before analysis. As with HPLC, tagging is necessary for spectrometric detection methods. Since CE uses a different separation method than HPLC, CE can be complementary to HPLC [1].

Many recent studies have utilized mass spectrometry, since it can rapidly produce results for profiling and characterizing glycans. However, quantitation is difficult, because ionization efficiency can depend on the structure of the oligosaccharide. Wada and colleagues have investigated HPLC with matrix-assisted laser desorption/ionization (MALDI) or electrospray ionization (ESI), but derivatization of the oligosaccharides is often employed to enhance sensitivity [12]. Derivatization of the samples results in better sensitivity, but has many disadvantages. For example, the method is time consuming, incomplete derivatization reactions can occur, and alterations to the structure of the
oligosaccharide can affect subsequent assays [15]. Also, derivatization reactions can require hazardous or toxic reagents such as cyanoborohydride. Finally, chemical methods for releasing glycans tend to reduce glycans, making attachments of a fluorophore to the reducing sugar carbon more difficult.

3) **Monosaccharide Analysis**

Carbohydrates can be broken down into monosaccharide constituents for further analysis. With HPAEC-PAD, monosaccharides can be identified and quantified at the femtomole level without needing any derivatization steps. The molar ratio of each of the monosaccharides can be obtained. The -OH groups of monosaccharides form oxyanions under alkaline conditions and form a hierarchy in terms of acidity [16]. To determine the bonding pattern of the monosaccharides, permethylation followed by bond cleavage can be performed. The –OH groups indicate where the bonds were and the –OCH₃ groups indicate where the –OH groups were [17].

4) **Summary of Methods to Date**

Even with all the new technology and methodology, it is still difficult to accurately quantify oligosaccharides. Analyses tend to give peak areas which depend on both structure and concentration. While glycan standards are available, they are typically expensive and in such small quantities that preparation of accurate concentrations are very difficult. Additionally, only a small fraction of biological glycans are commercially available. A recent study with HPLC with charged aerosol detection (CAD) has
demonstrated good sensitivity for the direct detection of non-derivatized oligosaccharides that is independent of their structural properties [15].

**Background on HPLC-CAD**

HPLC-CAD is a recent technique that is related to evaporative light-scattering detection. A diagram of an HPLC-CAD system is shown in Figures 4 and 5. As can be seen, the effluent from the HPLC column is nebulized by nitrogen gas, evaporated, and the dried particles are detected with positively charged nitrogen cations. Originally, the particles were charged by passage through a corona discharge region. However, more recently, the detector has been modified by using the charge left on the spray droplets that are produced in nebulization. Then the charged aerosol particles pass into an ion filter produced by a negatively charged rod and finally are detected by an electrometer. The non-volatile compounds give an electrical response based primarily on their mass concentrations. Charged aerosol detection (CAD) can be used to detect non-UV-absorbing sugars [15, 18, 19].
Figure 4. A generic aerosol-based detector for HPLC.

Figure 5. Schematic diagram of the electrical aerosol size analyzer (EAA).
HPLC-CAD is a good technique for the analysis of oligosaccharides because it is able to detect compounds that do not have chromophores or absorb UV light. CAD is referred to as a universal detector meaning that the analysis of oligosaccharides can be performed without any derivatization. Also, the magnitude of the response current is dependent upon the mass concentration and is not influenced much by analytical properties such as molar absorptivity or proton affinity. However, one disadvantage with CAD is that the buffer solutions in the mobile phase are restricted to volatile components. Also, the flow rate, the temperature of the mobile phase, and the composition of the eluent affect the CAD current \cite{15}. The effect of eluent composition causes gradient elutions to have an increasing or a decreasing sensitivity with time.

Also, a gradient elution will not have uniform response, due to differences in droplet size during nebulization and evaporation. The changes in organic solvent and water concentrations during the gradient elution affect the transport efficiency within the detector, causing a change in detector response. To correct for this error, a two dimensional calibration can be performed if standards are not available for the specific compound. An empirical model, which relates the mobile phase composition or retention time, analyte concentration, and detector response, can be determined \cite{20, 21}.

**Universal Calibration Method**

Traditionally, power fit calibration curves have been used (see Equation 1) for describing aerosol-based detector response \cite{22}.

\[ y = AC^b \]  

Equation 1
In Equation 1, ‘y’ is the peak area and ‘C’ is the concentration. Both ‘A’ and ‘b’ parameters are constant for given compounds, and are traditionally found by analyzing standards over a concentration range. Because the CAD is a universal detector, parameters ‘A’ and ‘b’ should not depend strongly on analyte structure, allowing one standard to be used for many analyzed compounds. However, the variety of different glycan structures requires the use of gradient elutions in which detector sensitivity changes. Both another group [21] and our research group [20] have developed a way to perform universal calibration while using a gradient elution. This involves analyzing a set of calibration standards over a range of eluent composition during elution [21] or retention times [20] so that empirical functions relating the ‘A’ and ‘b’ parameters to eluent composition or retention time can be derived. In our case, for unknown compounds, the ‘A’ and ‘b’ parameters can be determined based on the unknown compounds’ retention times, and the concentrations of unknown compound is calculated based on Equation 2.

\[ C = \frac{y}{A}^{\frac{1}{b}} \]  

Equation 2

The earlier work, performed by Noah Kiedrowski for his master’s thesis, used an amino column with calibration standards consisting of linear and cyclic glucose oligosaccharides [20]. The universal calibration method was found to be largely successful with most test compounds showing quantification errors of under 20%.
Traditionally, hydrophilic-phase chromatography using amine- and amide-bonded stationary phases have been believed to be one of the more effective columns for glycan separation. However, the method was limited in sensitivity due to column bleed while giving moderate resolution [20]. As can be seen in Figure 6, when Kiedrowski analyzed *Xenopus laevis* egg jelly glycans, he noticed that the chromatograms had elevated baselines and overlapping peaks which make quantification difficult.

Graphitized carbon columns have recently become more commonly used since they are easier to use and have high capacity and high efficiency. They have a homogenous adsorptive nature, and are rugged (are unaffected by strongly acidic or basic conditions and have low bleed characteristics) [23]. The retention on the porous graphitic carbon (PGC) column is based on dispersive interactions between the analyte-mobile phase and analyte-graphite surface. Retention generally increases as the hydrophobicity of the analyte increases. However, the retention is also based on charge-induced interactions of a polar analyte with the polarizable surface of the graphite, making accurate prediction of retention times difficult [24]. The use of a PGC column has given results with greater sensitivity and better resolution when applied to reduced glycans from biological samples [20] as shown in Figure 7. A 3 μm particle diameter, 100 x 4 mm Hypercarb PGC column was used for these studies. The small particle size packing material (3 μm diameter particles) makes this column a more efficient column (smaller plate height) than the amino column (5 μm diameter particles). However, Kiedrowski was not able to generate a universal calibration method using the PGC column.
**Figure 6.** Chromatogram of glycans from *X. laevis* egg jelly on an amino column. N. Kiedrowski.

**Figure 7.** Chromatograph of glycans from *X. laevis* egg jelly on a PGC column. N. Kiedrowski.
Independent Determination of Oligosaccharide Standards

While Kiedrowski was able to use HPLC-CAD with a universal calibration method to determine concentrations of test standards, a limitation for the application to test standards more structurally related to glycans was that an independent determination of the standard concentration was difficult [20]. Many such standards are too expensive to have sufficient quantities to weigh accurately in the preparation of standards. One alternative method is to use a spectroscopic assay to determine oligosaccharide concentrations. The phenol sulfuric acid procedure is an easy way for measuring neutral sugars in oligosaccharides, proteoglycans, glycoproteins, and glycolipids. It is a fast and reliable method that only requires a spectrophotometer for analysis [29].
OBJECTIVES

The quantification of oligosaccharides has still proven to be a challenge. The analysis with a HPLC-CAD system has provided a universal detection method with good sensitivity for oligosaccharides. The goal of this graduate research project was first to improve quantification of oligosaccharides by analysis on the HPLC-CAD using a PGC column. This column showed promise in preliminary studies in having greater sensitivity and resolution than the amino column used by Kiedrowski [20]. Specific goals of this project include measuring the performance of the PGC column method and applying this method to two well-known biological glycoproteins, immunoglobulin G (IgG) and ribonuclease B (RNase B), to obtain quantitative results about the oligosaccharides present.
MATERIALS AND METHODS

Methodology Development in Quantification of Oligosaccharides

1) Phenol-Sulfuric Acid Analysis

Previous results of test standards obtained by Noah Kiedrowski were inconsistent with the manufacturer’s label. Therefore, a phenol-sulfuric acid assay was performed to independently estimate how much sugar was present in the test standards. While this method does not distinguish between different types of sugars, different monosaccharides give different responses, requiring multiple monosaccharide calibration standards to be used. For example, to test the response of sucrose, which is a disaccharide composed of glucose and fructose, calibration was done using an assumed linear response with slope and intercept values from the averages found for glucose and fructose. Similar weighted averages were used for other oligosaccharides based on the monosaccharide composition of each oligosaccharide. Samples were weighed out, dissolved in water, and 25 µL of the sample was treated with 500 µL of 4% phenol and 2.5 mL of 96% sulfuric acid, and analyzed on a Unico (Dayton, NJ) UV-VIS spectrophotometer at 490 nm.

2) HPLC-CAD

Separation was performed on an Agilent (Santa Clara, CA) 1100 HPLC system equipped with a binary pump operated using Chem Station software and a variable wavelength detector in series with a built in-house CAD system. The CAD system was modified from the system described in Dixon and Peterson [18]. The main parts of a CAD include a nebulizer, spray chamber, an oven and an Electrical Aerosol Size
Analyzer (EAA, TSI Inc., Shoreview, MN). Figure 8 shows the nebulizer, spray chamber and oven used by the CAD. The effluent that exits the HPLC is nebulized in a Meinhard (Golden, CO) nebulizer leading to a Glass Expansion (West Melbourne, Australia) spray chamber. The spray chamber serves to reduce excessive and larger spray droplets. Remaining droplets are then passed on to the oven, where they are evaporated before reaching the EAA. The spray process also imparts charges of both polarities on many droplets through a process termed spray electrification [26]. This charge remains with most droplets when they evaporate. While the EAA is designed to charge particles through production of charged molecules in a corona discharge region and attachment of these charged molecules to the aerosol particles, the corona discharge was disconnected for this work. After the aerosol particles pass through the corona discharge region of the EAA, they then pass through a cylindrical electric field region, which is designed to only allow the larger positively charged particles to pass. In this electric field region, most of the negatively charged particles become repelled from a negatively charged rod hitting an outer wall and being removed, while the larger positively charged particles pass through to the electrometer. The ion voltage on the EAA can be adjusted to increase or decrease the size of the positively charged particles and output signal as necessary. The CAD is described in more detail by Abhyankar [25].

Separations were performed using a 3 µm diameter packing material, 4 mm diameter x 100 mm length Hypercarb PGC column (Thermo Fisher Scientific, Waltham, MA). This column showed promise in preliminary studies, having greater sensitivity and resolution than conventional columns, as discussed in the background section [20].
Injection volumes of 20 μL at an HPLC flow rate of 1 mL/min were used for initial standard tests. With solvent A as nanopure H₂O (CSUS CIMERA lab) and solvent B as acetonitrile (ACN; HPLC grade, Acros, Geel Belgium), the elution method was a gradient elution of 5% to 25% B in 20 minutes, then held at 25% B for 5 minutes, and then decreased linearly to 5% B over 5 minutes. The injection volume was changed to 5 μL when analyzing standards dissolved in 50% or greater ACN concentrations because sample dispersion in the column was observed otherwise.

Initially, a gradient elution with increasing acetonitrile of 5% to 65% was used to separate the standards used for calibration. However, 65% B was not needed to elute out
all compounds. Based on those results, a gradient method with increasing acetonitrile from 5% to 25% over 30 minutes achieved separation efficiently, as discussed in the Results and Discussion section. EAA ion voltages of -300, -225, and -150 mV were used to test sensitivity, linearity, and detectable concentration range of maltotetraitol under various conditions.

3) **Calibration standards**

Reduced oligosaccharides with degrees of polymerization ranging between 2 and 8 were used as calibration standards (see Table 1). These standards were obtained from Sigma Aldrich (St. Louis, MO) and Supelco (Oligosaccharide Kit and Sugar Alcohol Kit, Bellefonte, PA) in powder form and brought into solution in water. Solutions of mixed analytes were made at 0.2, 1, 4, 10, and 25 μg/mL in water. Galactitol and maltitetraitol were originally included as calibration standards.

<table>
<thead>
<tr>
<th>Degree of Polymerization</th>
<th>Purity (%)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltitol</td>
<td>2</td>
<td>99.5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>2</td>
<td>-</td>
</tr>
<tr>
<td>Melezitose</td>
<td>3</td>
<td>99.8</td>
</tr>
<tr>
<td>Stachyose</td>
<td>4</td>
<td>98.0</td>
</tr>
<tr>
<td>α-Cyclodextrin</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>β-Cyclodextrin</td>
<td>7</td>
<td>≥98</td>
</tr>
<tr>
<td>γ-Cyclodextrin</td>
<td>8</td>
<td>≥98</td>
</tr>
</tbody>
</table>

*Certified standard
4) Reduction of Test Oligosaccharides Using Ion Exchange Column

Five hundred µg of reducing oligosaccharides were reduced with 1 M sodium borohydride (Fischer Scientific, Waltham, MA) in 0.1 M sodium hydroxide (Fischer Scientific, Waltham, MA) solution. These samples were allowed to react for 24 hours at 45°C on a heating block. Then the samples were passed through a strongly acidic cation exchange resin of Dowex 50W-X4 (J.T. Baker Chemical Co., Phillipsburg, NJ) and flushed into 50 mL volumetric flasks using water to obtain concentrations of 10 µg/mL, prior to analysis on the HPLC. The purpose of the cation exchange column was to neutralize the salts of the NaBH₄ and NaOH after the reaction. Because this cation exchange method required a large amount of oligosaccharide starting material followed by a large dilution, another method was tested to reduce the amount needed.

5) Reduction of Test Oligosaccharides Using Solid Phase Extraction

Ten µg of reducing oligosaccharides were reduced with 1 M sodium borohydride in 0.1 M sodium hydroxide solution. These samples were allowed to react for 24 hours at 45°C on a heating block. The samples were then passed through 150 mg/4mL Carbograph solid phase extraction (SPE) cartridges (Grace Davison Discovery Science, Deerfield, IL). Prior to loading the samples, the SPE cartridges were preconditioned with 1 mL of 25:75 ACN:H₂O and then equilibrated with 1 mL of H₂O. After passing the samples through the columns, they were washed with 1 mL of H₂O, eluted with 1 mL of 25:75 ACN:H₂O, and analyzed by HPLC-CAD. Band broadening was observed during the analysis of these samples due to the solvent being a stronger eluter. Therefore, the injection volume was decreased to 5 µL. To further improve the chromatography, the samples were evaporated
and reconstituted with H$_2$O following the elution step for SPE, and then analyzed by HPLC using 20 µL injections.

6) **Reducing Contaminants**

All injections vials and caps were soaked in H$_2$O for approximately 24 hours and then air-dried prior to use. The volumes of preconditioning, equilibrating, and washing solutions were increased from 1 mL to 5 mL for SPE columns.

7) **Reducing the Tailing on Chromatogram Peaks**

To reduce the amount of tailing of later-eluting compounds, the organic solvent of the mobile phase was changed from 100% ACN to 25% tetrahydrofuran (THF, Sigma Aldrich, St. Louis, MO) in H$_2$O. A gradient elution of 5% to 55% of 25% THF in H$_2$O in 25 minutes was used.

**Analysis of Test Glycoproteins**

Previously, Kiedrowski used NaBH$_4$ and NaOH for the cleavage of O-linked oligosaccharides. However, it was discovered later that this method is not optimal for N-linked oligosaccharides. Even though this may result in decreased yields of glycans, this method was still utilized. IgG (Figure 9) and RNase B (Figure 10) were obtained from Sigma Aldrich and analyzed using the SPE method. Based on the SPE work up method used on test standards, roughly ten µg of each glycoprotein oligosaccharide was needed for clear detection. With IgG having 2.2% glycan by mass and assuming the major glycans constitute roughly 20% of oligosaccharide mass, 2 mg of starting glycoprotein was calculated as a needed amount. The same amount of starting material of RNase B,
which contains about 10% glycan by mass, was used. The glycoproteins were dissolved in 150 mM NaCl. Similar methods as those described previously for standards were used to cleave and reduce the oligosaccharides: NaBH₄ was added to the dissolved glycoproteins, heated at 45°C for 24 hours, and run through Carbograph SPE columns. Then the samples were lyophilized and reconstituted before analysis on the HPLC-CAD. A gradient elution of 5% to 25% B was used, but the run time was increased to 60 minutes to increase the separation of compounds and the voltage was increased to -150 V to increase the signal for both IgG and RNase B.

Figure 9. Immunoglobulin G
http://www.umass.edu/microbio/rasmol/igg_w.gif
Figure 10. Ribonuclease B
RESULTS AND DISCUSSION

Phenol Sulfuric Acid Assay

The results from the phenol sulfuric acid analysis are shown in Table 2. The percent errors obtained by Kiedrowski, calculated as the difference between the universal response-derived concentration and the weighed concentration generally are very similar to those obtained by the phenol sulfuric acid assay. Errors of less than 4% were observed for raffinose, maltotetraose, maltohexaose, and maltoheptaose. However, some oligosaccharide results were inconsistent. Kiedrowski found the percent error for maltooctaose, isomaltotriose and mannopentaose to be -10.7%, -10.8% and -14.3%, respectively, while I obtained 2.91%, 0.86% and 6.60%, respectively. Because maltooctaose was previously found to not be very pure (it contained observable concentrations of shorter chain glucose oligomers) [Dixon, personal communication], the greater error in the HPLC-CAD method could have resulted from other glucose oligomers contributing to the phenol sulfuric acid signal but not to the HPLC-CAD response. The lower HPLC-CAD response for isomaltotriose and mannopentaose indicate either lower than expected sensitivity for those oligosaccharides, or possibly the presence of other contaminant carbohydrates that did not elute from the column in the standards. The phenol sulfuric acid assay allows a secondary method to determine total oligosaccharide concentration.
Table 2. Results associated with phenol sulfuric acid analysis.

<table>
<thead>
<tr>
<th></th>
<th>Expected Mass (µg)</th>
<th>Experimental Mass (µg)</th>
<th>% Errors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>40.0</td>
<td>39.447</td>
<td>-1.38</td>
</tr>
<tr>
<td>Melezitose</td>
<td>40.0</td>
<td>42.011</td>
<td>5.03</td>
</tr>
<tr>
<td>Raffinose</td>
<td>40.0</td>
<td>37.098</td>
<td>-7.26</td>
</tr>
<tr>
<td>Isomaltotriose</td>
<td>40.0</td>
<td>40.342</td>
<td>0.86</td>
</tr>
<tr>
<td>Maltotetraose</td>
<td>40.0</td>
<td>38.922</td>
<td>-2.70</td>
</tr>
<tr>
<td>Maltohexaose</td>
<td>40.0</td>
<td>40.399</td>
<td>0.997</td>
</tr>
<tr>
<td>Maltoheptaose</td>
<td>40.0</td>
<td>37.958</td>
<td>-5.10</td>
</tr>
<tr>
<td>Maltooctaose</td>
<td>40.0</td>
<td>41.163</td>
<td>2.91</td>
</tr>
<tr>
<td>Mannopentaose</td>
<td>40.0</td>
<td>42.639</td>
<td>6.60</td>
</tr>
</tbody>
</table>

Chromatography

Both reducing oligosaccharides and non-reducing oligosaccharides (sugar alcohols or “food sugars” - the term given here for oligosaccharides containing two anomeric carbons linked together and cyclodextrins) were investigated for use as calibration standards. Food sugars such as sucrose, melezitose, and stachyose are non-reducing but not sugar alcohols. The food sugars were readily available (purchased as a kit containing glucose oligomers used by Kiedrowski [20]). While reducing oligosaccharides such as those used by Kiedrowski [20] for calibration gave single peaks in amino column separations, the same compounds gave multiple peaks due to separation of α and β anomers, making calibration difficult. Figure 11 shows the α and β forms of glucose. The hydroxyl groups on carbon 1 of the glucose molecule have different stereochemistry, and on some chromatography columns will have differing retention
times. As shown in Figure 12, maltotriose produces two peaks in the reducing form and one peak following reduction to maltotriitol with alkaline borohydride (see Figure 13 for the chemical reaction.) The two peaks for maltotriose show the separation of the α and β anomers. The sum of the peak areas for the maltotriose anomers is approximately the same as the peak area of maltotriitol (within 8%), indicating efficient conversion to the reduced form and similar responses for each compound. The data associated with the reduction is shown in Table 3. In order to reduce sugars, NaBH₄ was added followed by a cation exchange cleanup step to remove the excess borohydride. This methodology was shown to be valid for oligosaccharides with degrees of polymerization (DP) ranging from 3 to 8. The “double” peaks given by reducing sugars left three options for a universal calibration: calibrating with multiple peaks, switching to the use of non-reducing sugars, or employing strategies to collapse double peaks into single peaks. The use of double peaks in calibration standards probably would have decreased accuracy (requiring accurate integration of two peaks). A past attempt was made to collapse double peaks by increasing the column temperature, increasing the conversion rate between the anomers [Dixon, unpublished data]. However, even at moderately high temperatures, the collapse to a single sharp peak was not complete.
Figure 11. The α and β anomers of glucose.
The positions of the –OH group at carbon 1 do not have the same stereochemistry.

Figure 12. Chromatograms of maltotriose and maltotriitol analyzed on the PGC column. The maltotriitol (the reduced form of maltotriose) is traced with a solid line and reducing maltotriose is traced in the dotted line. The x-axis is time (min) and y-axis is response in mV.
Table 3. Data obtained from reduction of oligosaccharides. Areas are given for reducing oligosaccharides and their corresponding reduced forms.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Combined α and β Peak Areas (mV)</th>
<th>Avg Peak Area of Reduced Form (mV)</th>
<th>Difference #</th>
<th>% Conversion</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maltotriose</td>
<td>2005</td>
<td>2159</td>
<td>154</td>
<td>108</td>
<td>0.668</td>
</tr>
<tr>
<td>Maltotetraose</td>
<td>2036</td>
<td>1975</td>
<td>-61</td>
<td>97</td>
<td>8.19</td>
</tr>
<tr>
<td>Maltohexaose</td>
<td>2432</td>
<td>2246</td>
<td>-186</td>
<td>92</td>
<td>13.9</td>
</tr>
<tr>
<td>Maltooctaose</td>
<td>2140</td>
<td>2257*</td>
<td>117</td>
<td>105</td>
<td>-</td>
</tr>
</tbody>
</table>

*Only one run

#Reduced area – non-reducing area

Figure 13. The reduction of maltotriose using sodium borohydride. The aldehyde group of carbon 1 is reduced to an alcohol group. The figure on the left is β-maltotriose.

The ion voltage can be changed on the EAA to accommodate a greater range of sample quantity. Ion voltages of -150, -225, and -300 volts were tested using the calibration standards to see which would be best for our sample range. At -150V, results showed higher peak areas for the same sample concentration, and greater signal-to-noise ratios. At -300 V, results showed smaller peak areas for the same sample concentration and smaller signal-to-noise ratios. The data associated with the ion voltage optimization is shown in Table 4. From a sensitivity perspective, the -150 V is the optimal ion voltage.
due to the greatest signal-to-noise ratio and lowest limit of detection. However, -225 V was chosen for analyses so that peaks would not go off scale at higher concentrations (greater useful concentration range). With all 3 ion voltages of the EAA, excellent calibration power fits were observed with $r^2$ values of ≥0.9980 (Figure 14). At -225 V, a true linear response is observed based on a ‘b’ value of nearly 1.

Table 4. Data associated with ion voltages of the EAA.

<table>
<thead>
<tr>
<th></th>
<th>Peak Areas (mV) With the EAA at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-150 V</td>
</tr>
<tr>
<td>Maltotetraitol</td>
<td></td>
</tr>
<tr>
<td>25 µg/mL</td>
<td>-150 V</td>
</tr>
<tr>
<td>10 µg/mL</td>
<td>4421.7</td>
</tr>
<tr>
<td>4 µg/mL</td>
<td>1907.0</td>
</tr>
<tr>
<td>1 µg/mL</td>
<td>597.2</td>
</tr>
<tr>
<td>0.2 µg/mL</td>
<td>214.3</td>
</tr>
<tr>
<td>S/N Ratio at 0.2 µg/mL</td>
<td>39</td>
</tr>
</tbody>
</table>
| LOD at 0.2 µg/mL | 5.1 ng/mL or 0.10 ng  | 5.7 ng/mL or 0.11 ng  | 10 ng/mL or 0.21 ng
In order to quantify the concentration of samples, a linear calibration of non-reducing oligosaccharide standards ranging in degree of polymerization (DP) from 1 to 4 and non-reducing cyclic oligomers, α-, β-, γ-cyclodextrin (DP 6, 7, and 8 respectively) were prepared at 0.2, 1, 4, 10 and 20 µg/mL in 100% water. A chromatogram of the 20 µg/mL standard is shown in Figure 15. From the analysis of a blank sample, contaminants co-eluted with galactitol and maltotetraitol; these were therefore not used as part of the calibration proceeding forward. Previously, 25 µg/mL was used as the highest end of the calibration standards. However, during some analyses, the peaks for stachyose and α-cyclodextrin went off scale. The highest concentration of the calibration standards was lowered to 20 µg/mL to resolve this problem. Most of the standards gave sharp and narrow peaks, except for β- and γ-cyclodextrin. Tailing was observed for β- and γ-
cyclodextrin (see Figure 15 and asymmetry values in Table 5), and tails are not consistent among the different chromatograms. This may be due to differing column conditions between day-to-day use. Also, there is wide separation between the standards because the method was not optimized for maximum efficiency. The glycans of glycoproteins are anticipated to need a longer separation period to achieve separation of peaks.

The peak areas were plotted against the concentrations of the calibration standards to give standard curves as shown in Figure 16. A table of data associated with the chromatogram given can be seen in Table 5. The A and b terms of the standard calibration curves were plotted against retention times to obtain polynomial curves in Figures 17 and 18.

Figure 15. Chromatogram of DP1 to 8 non-reducing glucose oligomers. Galactitol, maltitol, sucrose, melezitose, stachyose, and α-, β-, γ-cyclodextrins at concentration of 20 μg mL⁻¹ were analyzed on the PGC column.
Table 5. Data associated with the chromatogram shown in Figure 4.

<table>
<thead>
<tr>
<th></th>
<th>Purity (%)</th>
<th>Retention Time (min.)</th>
<th>Peak Area (mV)</th>
<th>Peak Width (min.)</th>
<th>Peak Asymmetry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactitol</td>
<td>99.9</td>
<td>1.680</td>
<td>240.6</td>
<td>0.1121</td>
<td>1.176</td>
</tr>
<tr>
<td>Maltitol</td>
<td>99.5</td>
<td>2.533</td>
<td>145.5</td>
<td>0.0967</td>
<td>0.692</td>
</tr>
<tr>
<td>Sucrose</td>
<td>-</td>
<td>3.190</td>
<td>146.9</td>
<td>0.1001</td>
<td>0.707</td>
</tr>
<tr>
<td>Melezitose</td>
<td>99.8</td>
<td>3.976</td>
<td>149.1</td>
<td>0.1015</td>
<td>0.721</td>
</tr>
<tr>
<td>Stachyose</td>
<td>98.0</td>
<td>9.004</td>
<td>169.9</td>
<td>0.1043</td>
<td>0.693</td>
</tr>
<tr>
<td>α-cyclodextrin</td>
<td>-</td>
<td>10.096</td>
<td>189.6</td>
<td>0.1136</td>
<td>0.703</td>
</tr>
<tr>
<td>β-cyclodextrin</td>
<td>≥98</td>
<td>12.945</td>
<td>109.7</td>
<td>0.2674</td>
<td>0.422</td>
</tr>
<tr>
<td>γ-cyclodextrin</td>
<td>≥98</td>
<td>14.388</td>
<td>174.6</td>
<td>0.1799</td>
<td>0.469</td>
</tr>
</tbody>
</table>

Figure 16. Power-fit standard calibration plots of non-reducing oligosaccharides.
Figure 17. Plot of A-terms as a function of retention times. The curve shows a polynomial fit of A-terms derived from the power fits (e.g. shown in Figure 16).

Figure 18. Plot of b-terms as a function of retention times. The curve shows a polynomial fit of b-terms derived from the power fits (e.g. shown in Figure 16).
Column Comparison

The analysis of the non-reducing oligosaccharide standards with optimized ion voltages on the PGC column was well fitted (Figure 16) and demonstrated $r^2$ values of $\geq 0.9917$. With the amino column, an increasing baseline is observed, resulting from increasing bleed of the stationary phase as the percent water increases during the run (Figure 19. A and B). At 8 minutes, the peak width for DP3 was 0.223 minutes [20]. With the PGC column, the peaks are sharper and narrower (Figure 19. C and D). At 8 minutes, the peak width for DP4 was 0.1243 minutes. The peak width for the PGC column was

![Figure 19](image_url)

Figure 19. Comparison of signal to noise ratios. Top plots (A and B) for the amino column with standards at 1 µg/mL and bottom plots (C and D) are for PGC column with standards at 0.2 µg/mL. Chromatogram A (glucose) and C (maltotetraitol) show the best signal to noise ratios for each column while B (maltooctaose) and D (β-cyclodextrin) show the worst signal to noise ratios.
about two times less than that of the amino column and has nearly two times the peak capacity. The baseline is low and does not rise as much through the run. The detection limit was found to be about 0.2 ng, while previous results showed the amino column had a detection limit of about 1 ng. Figure 19 shows the signal to noise ratios of the PGC and amino column.

**Reduction of Test Oligosaccharides**

As assessed using HPLC with the PGC column, the reduction of unreduced oligosaccharides was a success with near 100% reduction using NaBH₄. Conversion ranged between 92% - 108% for four oligosaccharides tested. The conversion is calculated by comparing the peak areas of the reduced oligosaccharide with the sum of the two peaks produced by unreduced oligosaccharides. Results are shown in Table 3. Good precision was observed from the low average % relative standard deviation (RSDs) of 7.59 from test standards run in duplicate.

The PGC column has demonstrated improvements in the analysis of oligosaccharides by providing increased sensitivity and linearity over the amino column. Given the promising results of this analysis, it was hypothesized that it should be possible to accurately quantify biological samples that contain very small amounts of glycans.

Therefore, a set of test oligosaccharides were prepared at 10 μg/mL, reduced with NaBH₄ and then passed through a cation exchange column, as described in the methods. Analysis was performed on HPLC using the PGC column with 20 μL injections. The results of this experiment are shown in Table 6. The percent error associated with the
samples ranged from -24.7 to 2.7%. The largest percent error occurred for galactose. Galactose was weakly retained on the PGC column, and its peak areas were influenced by co-eluting contaminants that also appeared in the blank. Maltose showed a very high percent recovery. This may be due to manufacturer error in reporting the concentration.

Table 6. Results of test standards – Set I. These were purified using a cation exchange column and run on a PGC column.
* Recovery based on peak area comparisons to reduced standard.
# Recovery based on peak area comparison to sum of anomeric peaks in unreduced standard.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Purity (%)</th>
<th>Retention Time (minutes)</th>
<th>Average Peak Area (mV)</th>
<th>Average Peak Area A term</th>
<th>Average Peak Area b term</th>
<th>Conc. μg/mL</th>
<th>% RSD</th>
<th>% Recovered</th>
<th>% Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>-</td>
<td>1.8495</td>
<td>1549.60</td>
<td>182</td>
<td>1.06</td>
<td>10</td>
<td>9.32</td>
<td>102.5*</td>
<td>-24.7</td>
</tr>
<tr>
<td>Maltose</td>
<td>-</td>
<td>2.634</td>
<td>2016.75</td>
<td>178</td>
<td>1.08</td>
<td>9.9</td>
<td>1.44</td>
<td>160.1*</td>
<td>-4.39</td>
</tr>
<tr>
<td>Maltotriose</td>
<td>98.0</td>
<td>5.425</td>
<td>2137.55</td>
<td>168</td>
<td>1.14</td>
<td>9.9</td>
<td>1.47</td>
<td>105.4*</td>
<td>-5.49</td>
</tr>
<tr>
<td>Maltotetraose</td>
<td>96.0</td>
<td>7.996</td>
<td>2427.35</td>
<td>222</td>
<td>1.07</td>
<td>10</td>
<td>7.96</td>
<td>93.6*</td>
<td>-6.35</td>
</tr>
<tr>
<td>Maltohexaose</td>
<td>98.0</td>
<td>10.808</td>
<td>2446.95</td>
<td>239</td>
<td>1.08</td>
<td>10</td>
<td>1.62</td>
<td>81.7*</td>
<td>-13.2</td>
</tr>
<tr>
<td>Maltooctaose</td>
<td>76.5</td>
<td>12.499</td>
<td>1957.75</td>
<td>176</td>
<td>1.2</td>
<td>7.65</td>
<td>1.49</td>
<td>112.9*</td>
<td>-2.78</td>
</tr>
<tr>
<td>Isomaltotriose</td>
<td>98.0</td>
<td>5.0575</td>
<td>2300.05</td>
<td>168</td>
<td>1.13</td>
<td>9.8</td>
<td>0.54</td>
<td>102.0*</td>
<td>2.7</td>
</tr>
</tbody>
</table>

Even though the analysis method on the HPLC-CAD showed good results, there still are some disadvantages. Sugar standards in the alcohol form are expensive and not as readily available as reducing sugars. Thus, the analysis with the PGC column does not include a full range of compounds between DP 1 and 8. Another disadvantage of using
the PGC column is that reducing sugars have to be reduced to alcohol forms before analysis. However, a reproducible method of reduction has been utilized, and sodium borohydride reduction is a common method incorporated into chemical cleaving glycans from glycoproteins. Contamination was more of an issue than with the amino column (see, for example, the unidentified peaks in Figure 4D).

Tailing was observed for β- and γ-cyclodextrins (DP7 and DP 8, respectively) on the PGC column, particularly at higher concentrations, which makes quantitation difficult. Tailing results in less well integrated peaks, causes more overlap between other peaks, and can affect ‘A’ and ‘b’ parameters determined in the calibration process. To reduce the tailing that occurs with the later eluting peaks, the solvent used in the mobile phase was changed from acetonitrile to tetrahydrofuran. The gradient elution was changed to 5% to 65% of 25% THF in H₂O in 30 minutes. The results of this analysis showed promising results with less tailing observed for the cyclodextrin compounds. THF could be considered as a replacement solvent if tailing becomes worse.

Another disadvantage for this analytical procedure is that it required a large amount of starting glycoprotein material. However, biological samples have a relatively low percentage of oligosaccharides. Therefore, the amount of starting material must be minimized before analysis of biological samples can reasonably begin. Towards this end, carbograph SPE columns were utilized to minimize the amount of oligosaccharide starting material needed. Test oligosaccharides were reduced with NaBH₄ and trapped by SPE in 25:75 ACN:H₂O. Preliminarily, these samples were directly injected onto the
HPLC using the same method above. However, the resulting chromatograms had poor peak shape. Therefore, the injection volume was decreased to 5 μL to resolve the issue. The peak shape became much better. To further improve the chromatography, the samples were evaporated and reconstituted with H₂O following the elution step for SPE, and then analyzed by HPLC using 20 μL injections. This method showed less error and greater reproducibility (analyzed in triplicate) than those without evaporation. The data is shown in Table 7. Manufacture error in labeling the sample containers may have resulted in inaccurate results.

Table 7. Results of reduced test standards – Set II. These were purified on a carbograph SPE column and run on a PGC column.

<table>
<thead>
<tr>
<th>Compound Reduced</th>
<th>Retention Time (min)</th>
<th>Average Area (mV)</th>
<th>A term</th>
<th>b term</th>
<th>Conc. (μg/mL)</th>
<th>Calc. Conc. (μg/mL)</th>
<th>% RSD</th>
<th>% Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isomaltriose</td>
<td>5.109</td>
<td>2454.6</td>
<td>196.0</td>
<td>1.170</td>
<td>9.8</td>
<td>8.67</td>
<td>13.95</td>
<td>-11.561</td>
</tr>
<tr>
<td>Maltotetraose</td>
<td>8.095</td>
<td>2690.0</td>
<td>208.2</td>
<td>1.176</td>
<td>9.6</td>
<td>8.82</td>
<td>1.14</td>
<td>-8.151</td>
</tr>
<tr>
<td>Maltopentaose</td>
<td>9.776</td>
<td>1976.3</td>
<td>92.4</td>
<td>1.379</td>
<td>9.64</td>
<td>9.21</td>
<td>9.61</td>
<td>-4.461</td>
</tr>
<tr>
<td>Maltohexaose</td>
<td>10.843</td>
<td>2866.1</td>
<td>194.0</td>
<td>1.217</td>
<td>9.8</td>
<td>9.14</td>
<td>4.16</td>
<td>-6.745</td>
</tr>
<tr>
<td>Maltoheptaose</td>
<td>11.592</td>
<td>2233.3</td>
<td>186.0</td>
<td>1.234</td>
<td>9.4</td>
<td>7.49</td>
<td>4.24</td>
<td>-20.300</td>
</tr>
<tr>
<td>Maltooctaose</td>
<td>12.183</td>
<td>1829.9</td>
<td>178.3</td>
<td>1.250</td>
<td>7.65</td>
<td>6.44</td>
<td>7.18</td>
<td>-15.797</td>
</tr>
</tbody>
</table>

Using the Carbograph SPE columns followed by evaporation and reconstitution showed good accuracy and reproducibility while greatly reducing the amount of starting material needed. The cation exchange column used 500 μg starting material, while the SPE column only used 10 μg. The small volumes needed for the SPE column makes
analysis of glycoproteins possible. The %RSD is somewhat higher than analysis with
greater volumes, but this is expected due to more variable losses when using smaller
samples, smaller volumes, and more steps. The greater % errors seen with the SPE
column indicate greater losses. The compounds with the greatest negative % errors are
similar to the results of Kiedrowski [20]. Also, tailing was still observed from the later
eluting compounds.

**Analysis of Test Glycoproteins**

The simple glycoproteins IgG and RNase B were tested with similar methods as
the test standards. The run time was increased to 60 minutes to increase the separation of
compounds, and the voltage was increased to -150 V to increase the signal. Standards
were also analyzed at the same time. As mentioned in the Materials and Methods Section,
the cleavage and reduction method used was designed for O-linked glycoproteins and
may have resulted in incomplete cleavage of these N-linked glycoproteins.

IgG is a more complicated glycoprotein and resulted in much more complicated
chromatograms. The chromatogram (Figure 20) showed multiple overlapping peaks, and
the baseline was very noisy. RNase B showed good chromatographic results, with fewer
peaks that were more resolved (Figure 21). The greater noise for the IgG is expected
based on a lower % glycan mass in IgG compared to RNase B. Data associated with the
analysis of IgG and RNase B are shown in Table 8 and 9, respectively. The percentage of
each peak from the total amount of glycan is calculated, along with the concentrations.
However, the identity of each peak cannot be determined. For RNase B, Thaysen-
Andersen and colleagues observed 5 main peaks using normal phase HPLC [30]. These peaks included \( \text{Man}_5\text{GlcNAc}_2 \) to \( \text{Man}_9\text{GlcNAc}_2 \). Abundances for each compound are listed: 47.3% \( \text{Man}_5\text{GlcNAc}_2 \), 27.7% \( \text{Man}_6\text{GlcNAc}_2 \), 7.9% \( \text{Man}_7\text{GlcNAc}_2 \), 13.5% \( \text{Man}_8\text{GlcNAc}_2 \), and 4% \( \text{Man}_9\text{GlcNAc}_2 \) [30]. Peaks 2 through 6 in Figure 21 appear to be associated with the \( \text{ManGlcNAc} \) compounds. With IgG having ~2% glycan structure, 5 mg of starting oligosaccharide material should yield about 100 µg of glycan. IgG yielded 47.6 µg, approximately 1%. RNase B yielded 258 µg, approximately 5%.

Figure 20. Chromatogram of immunoglobulin G glycans. Run time was increased to 60 minutes and voltage was increased to -150 mV.
Table 8. Data associated with IgG chromatogram.

<table>
<thead>
<tr>
<th></th>
<th>Time (min)</th>
<th>Area (mV)</th>
<th>Height (mV)</th>
<th>Width (min)</th>
<th>Calculated Concentration (µg/mL)</th>
<th>% Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.96</td>
<td>800.1</td>
<td>13.3</td>
<td>0.7281</td>
<td>4.4</td>
<td>9</td>
</tr>
<tr>
<td>2</td>
<td>14.457</td>
<td>972.5</td>
<td>14.2</td>
<td>0.8085</td>
<td>5.0</td>
<td>11</td>
</tr>
<tr>
<td>3</td>
<td>25.794</td>
<td>405.6</td>
<td>7.2</td>
<td>0.6664</td>
<td>2.0</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>30.458</td>
<td>3309.3</td>
<td>108.6</td>
<td>0.4115</td>
<td>13.4</td>
<td>28</td>
</tr>
<tr>
<td>5</td>
<td>31.752</td>
<td>1137.3</td>
<td>25.3</td>
<td>0.5344</td>
<td>5.0</td>
<td>10</td>
</tr>
<tr>
<td>6</td>
<td>33.148</td>
<td>2658.4</td>
<td>37.7</td>
<td>0.8371</td>
<td>10.6</td>
<td>22</td>
</tr>
<tr>
<td>7</td>
<td>34.425</td>
<td>1751.3</td>
<td>26.6</td>
<td>0.7845</td>
<td>7.2</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>47.6 µg</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 9. Data associated with RNase B chromatogram.

<table>
<thead>
<tr>
<th>Peak</th>
<th>Time (min)</th>
<th>Area (mV)</th>
<th>Height (mV)</th>
<th>Width (min)</th>
<th>Calculated Concentration (µg/mL)</th>
<th>% Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>22.665</td>
<td>814.5</td>
<td>14.4</td>
<td>0.6705</td>
<td>3.9</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>26.297</td>
<td>19539.7</td>
<td>275</td>
<td>0.8511</td>
<td>73.4</td>
<td>29</td>
</tr>
<tr>
<td>3</td>
<td>28.299</td>
<td>20069.6</td>
<td>343.9</td>
<td>0.7149</td>
<td>72.7</td>
<td>28</td>
</tr>
<tr>
<td>4</td>
<td>31.545</td>
<td>3331.3</td>
<td>100.4</td>
<td>0.419</td>
<td>13.3</td>
<td>5</td>
</tr>
<tr>
<td>5</td>
<td>33.788</td>
<td>22939.8</td>
<td>398.9</td>
<td>0.6908</td>
<td>71.7</td>
<td>28</td>
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<tr>
<td>6</td>
<td>35.404</td>
<td>3522.6</td>
<td>55.3</td>
<td>0.7555</td>
<td>13.2</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>43.045</td>
<td>2590</td>
<td>45.4</td>
<td>0.6932</td>
<td>9.3</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>258 µg</td>
<td>-</td>
</tr>
</tbody>
</table>
CONCLUSIONS

The PGC column has proved to be a good column for analyzing oligosaccharides on the HPLC-CAD. The signal-to-noise ratio is fivefold better with the PGC column than the amino column that was previously used. The baseline was also much lower with the PGC column than the amino column. We can effectively reduce oligosaccharides into an alcohol form with sodium borohydride, collect them with a carbograph SPE column, and quantify them on the HPLC-CAD. The test oligosaccharides that were reduced and quantified showed very good precision and good accuracy, even with small quantities. The reduction process cleaved glycans from glycoprotein IgG and RNase B, but without quantitative results. Incomplete cleavage/reduction of the glycans may have resulted in lower % yields due to the use of a reduction method designed for O-linked rather than N-linked glycans. Analysis on the HPLC-CAD showed good promise for future quantification of peaks.

With this method, the oligosaccharides are reduced during cleavage from the glycoprotein in a single step as opposed to multiple steps needed for common derivatization reactions and can be used for subsequent analyses. Also, this method is sensitive enough to detect sugars at the sub-nanogram level. However, it is still not possible to accurately identify which compounds correspond to the peaks of a chromatogram for analyzing glycans of a glycoprotein.
FUTURE WORK

With an adequate method developed for the analysis of underivatized oligosaccharides on the HPLC-CAD, additional analyses may be performed to obtain glycan profiles of RNase B and IgG. First, the peaks obtained for both glycoproteins need to be further resolved before further analysis. Then, the fractions can be collected and further analyzed by mass spectrometry. MS will be able to identify the components that correspond to the peaks of the chromatogram. Also, more of these realistic test compounds (glycan-like structures) should be tested for better estimation of percent errors in quantification. Many standards do not have sufficient amounts and the masses are too low to weigh. The more realistic test compounds could be tested by phenol sulfuric acid assay if it can be used for smaller quantities. More robust methods may need to be tested for cleaving and reducing glycans from N-linked glycoproteins.

Since not all oligosaccharides are neutral species, a method for analyzing charged oligosaccharides using the HPLC-CAD should also be developed. Standards such as succinyl-β-cyclodextrin (Figure 22) and carboxymethyl-β-cyclodextrin (Figure 23) can be purchased. Similar methods can be applied for the analyses of the charged compounds, but an acidic modifier needs to be added to the eluent.
Figure 22. Succinyl-β-cyclodextrin.  
http://www.sigmaaldrich.com/structureimages/97/mfcdf0800297.gif

Figure 23. Carboxymethyl-β-cyclodextrin.  
http://www.cncyclodextrin.com/upfile/20101113/201011131615474291.jpg
References


[31] Unpublished Lillian Jaquinod work.