Method for Simultaneous Quantitation of Free Carrier Protein and Free Polysaccharide in Glycoconjugate Vaccines by High Performance Liquid Chromatography

By

Ian Hartzel, 2019

Submitted to the graduate degree program in Pharmaceutical Chemistry and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Master of Science

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Chair: Dr. John Stobaugh, Ph. D.

Co-Chair: Dr. Nathan Lacher, Ph. D.

Member: Dr. Susan Lunte, Ph. D.

Date Defended: 22 August 2019

The thesis committee for Ian Hartzel certifies that this is the approved version of the following thesis:

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Abstract

Disease caused by bacterial infections affect children and adults worldwide can be potentially life-threatening. Bacterial strains, such as *Staphylococcus aureus* and *Streptococcus pnueumoniae* (pneumococcus), express capsular polysaccharides on their surface, which act as a virulence factor for evading the immune system and are structurally unique depending on the serotype. Vaccines designed against the capsular polysaccharides have shown to be effective in reducing the incident rate of disease caused by bacterial injections. Glycoconjugate vaccines comprise large molecular mass bacterial capsular polysaccharides conjugated to a carrier protein, which helps activate T cells that trigger immunological memory functions. Un-conjugated (free) carrier protein and polysaccharide are critical attributes typically included in analytical testing for glycoconjugate vaccines and must be monitored throughout the vaccine development process. High levels of free polysaccharide or carrier protein may be indicative of poor conjugation efficiency or product degradation caused by manufacturing inconsistencies, formulation, or storage conditions.

In this work, we will present the development of an HPLC method to simultaneously quantitate free carrier protein and free polysaccharide in a glycoconjugate vaccine drug substance. This method would be developed for two *S. aureus* glycoconjugate drug substances, CP5-CRM_{197} and CP8-CRM_{197}. Initial work focused on using hydrophobic interaction chromatography (HIC) as the mode of separation by utilizing the often-ignored separation space prior to the void volume. However, the separation proved to be difficult in a 1D format due to the complexity of the sample mixture and the chromatographic behavior of the glycoconjugate. Therefore, a 2D-LC approach was taken to separate and quantitate the free carrier protein and free polysaccharide in the two *S. aureus* glycoconjugate drug substances. This work focused on the development of SEC and RPLC methods, 2D-LC instrumentation design, 2D-LC method development, and assay performance. Results were then compared to results from current technologies in place based on capillary electrophoresis (CE) for the quantitation of free carrier protein and free polysaccharide to assess the feasibility of supporting manufacturing process and formulation development studies. In addition, this methodology was explored as a potential platform technology for monitoring free carrier protein and polysaccharide in other glycoconjugate vaccine projects in the development pipeline.

Acknowledgements

Yan He

Heidi Holovics

James Carroll

Steve Kolodziej

Agilent Technologies, Inc.

Contents

1. CHAPTER 1: INTRODUCTION

1.1. OVERALL GOAL:

The overall goal of this research project is to develop a high-performance liquid chromatography (HPLC) method for determining the amount of free carrier protein and free polysaccharide in glycoconjugate vaccine samples. The practical applications of the developed technology include manufacturing process development support, stability support, and release testing. The performance of the method will be assessed per ICH guidelines and compared against current technologies for measuring these attributes. The method will then be explored and evaluated on several vaccine glycoconjugates as a novel, platform-based approach for monitoring free carrier protein and free polysaccharide levels.

1.2. BACKGROUND AND SIGNIFICANCE:

Numerous bacterial species, such as *Staphylococcus aureus* and *Streptococcus pnueumoniae* (pneumococcus), express capsular polysaccharides on their surface [1, 2]. Both encapsulated bacterial species can produce a range of capsular polysaccharides, each with differing structure and chemical properties, which the defines the capsular type. Capsular polysaccharides are typically water soluble, highly hydrated, and most often acidic. They are linear polymers comprised of a repeating subunit of one to six monosaccharides covalently linked to the cell surface and can have high molecular weights due to repetition of the subunit. The capsular polysaccharides form a thick, mucous-like layer around the bacteria. These characteristics of the capsular polysaccharides make them an important virulence factor. The capsular polysaccharides aid in preventing desiccation, adherence to surfaces, and the evasion of the immune system, specifically, protecting the bacteria and preventing phagocytosis and masking antigenic proteins on the bacterial surface. These infectious bacteria pose a significant health

threat to broad age groups throughout the population, especially in infants and the elderly. Therefore, prevention of these infections through vaccinations has the potential to significantly improve public health.

Early developed vaccines against *H. influenzae* type b (Hib), consisted of purified capsular polysaccharide type b, the most prevalent and virulent strain that causes meningitis, epiglottitis, septicemia, facial cellulitis, pneumonia, and arthritis [2]. Initial studies observed protection against infection greater than 90% in children 18 months to 5 years old, however, children between 12 and 18 months showed questionable protection and children under 12 months no protection. Polysaccharides alone fail to activate T cells and B cells that trigger immunological memory functions. Conjugate vaccines act through a T-cell dependent pathway that trigger immunological memory functions [3]. This immune response permits re-vaccination and generally can be boosted. As polysaccharide vaccines use a T-cell independent pathway, limited duration of efficacy (three to five years) is seen for pneumococcal diseases. For these advantages, conjugate vaccines can be arguable preferred over polysaccharide vaccines. Initial conjugate vaccines utilized carrier proteins derived from diphtheria (Dt) and tetanus (Tt) toxoids due to their well understood safety and immunological profiles. Currently, the list of carrier proteins has expanded to also include cross-reacting material 197 (CRM_{197}), protein D, and outer membrane protein complex (OMPC) [4]. CRM_{197} is a non-toxic mutant of diphtheria toxoid with a single amino acid substitution in the enzymatically active domain of the toxin and is widely used throughout the biotech industry in prophylactic and therapeutic vaccines. The introduction of conjugated vaccines against invasive pneumococcal disease (IPD), such as Prevnar-13, has demonstrated a significant reduction of incidences among children below 5 years old and adults

greater than 65 years old compared for the vaccinated serotypes [5]. This shows the importance of conjugated vaccines in reducing the prevalence of the disease.

Conjugate vaccines are created through multiple step conjugation chemical reactions to generate a glycoconjugate. A general approach to glycoconjugate manufacturing is illustrated in Figure 1. The basic steps consist of activation of a purified capsular polysaccharide (CP), conjugation of the activated polysaccharide to a carrier protein i.e. CRM_{197}, and then capping of the unconjugated activate sites on the polysaccharide. Due to the varying polysaccharide sizes and activation sites, final glycoconjugates can be a large, heterogenous product with of a wide distribution of molecular weights in the mega Dalton range. This results in a complex sample mixture where all the components have unique behavioral characteristics due to their molecular structure. These properties make developing analytics for monitoring critical quality attributes a major challenge.

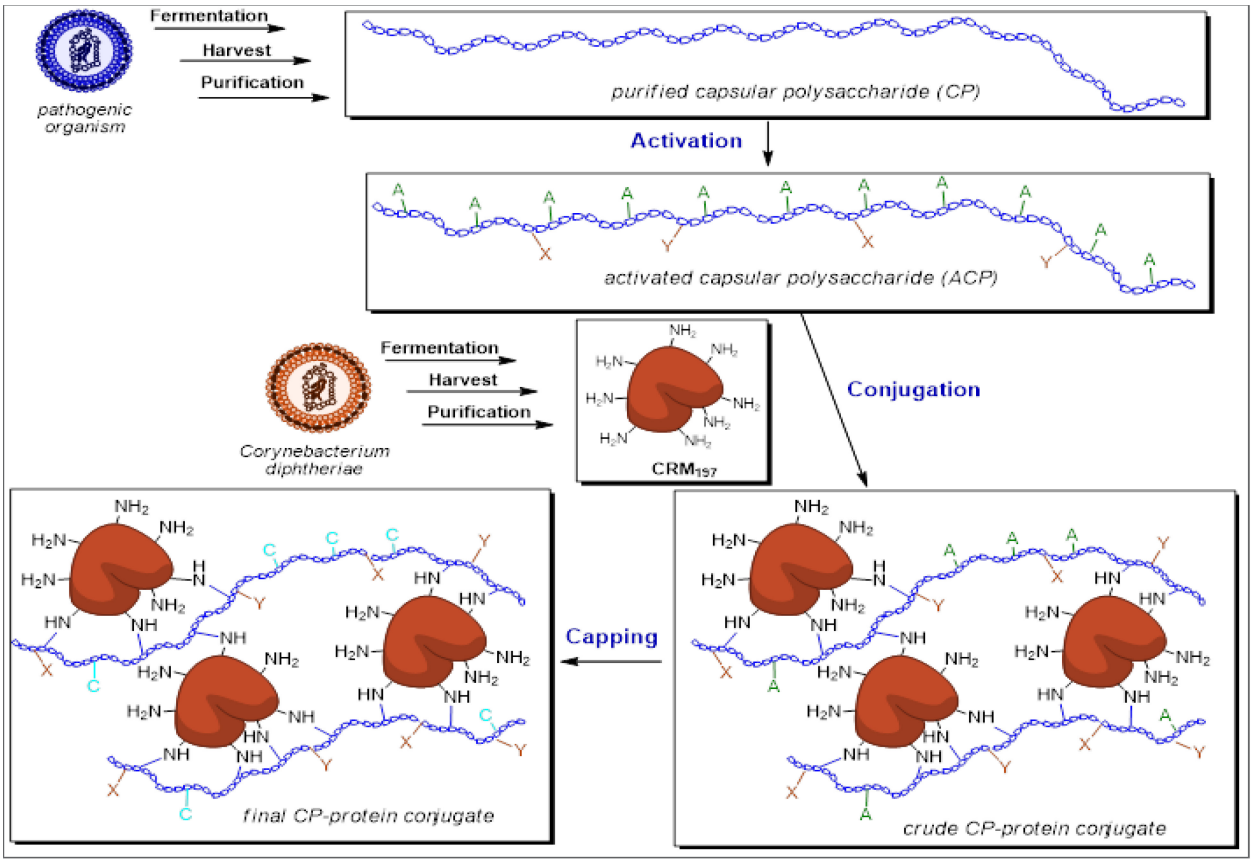

Figure 1: Flow diagram of a typical glycoconjugate manufacturing process

Manufacturing consistent glycoconjugate vaccines requires strong knowledge of the conjugation chemistry, detailed physicochemical characterization of the polysaccharide and carrier protein, and a well-defined and controlled manufacturing process. It is critical to monitor free carrier protein and free polysaccharide throughout the vaccine development process to achieve a consistent amount of conjugated polysaccharide in the vaccine, resulting in a desired and predictable immune response from the glycoconjugate. High levels of free polysaccharide or free protein can indicate poor conjugation efficiency or product degradation caused by manufacturing inconsistencies, formulation, or storage conditions. Monitoring these various species pose significant analytical challenges related to low throughput, multiple methods and technologies, sub-optimal resolution, and project-specific methods.

Various technologies and techniques have been implemented to measure free carrier protein and free polysaccharide. A recent review by Nunnally and Yao discussed the use of micellular electrokinetic chromatography (MEKC) as a routine method for quantitation of free protein for pneumococcal and meningococcal serotypes using Dt or Tt as carrier protein [6, 7]. The technique provides broad applications to different carrier proteins and polysaccharide serotypes and currently remains one of the best technologies for this application. However, limitations of the CE technology still exist, which include poor reproducibility and low throughput. Size exclusion chromatography (SEC) is another technique that has been attempted on glycoconjugates to monitor free polysaccharide and free carrier protein [8]. However, due to having similar distribution coefficients, separation of the free polysaccharide from the glycoconjugate has proved difficult using SEC alone. Also, for quantitation of free polysaccharide, solid-phased extraction (SPE) cartridges have been used [9]. These cartridges require several washing steps followed by an additional analysis by ionic chromatography. SPE is only utilized for free polysaccharide quantitation. The techniques described are suitable for the intended purpose, but each has a limitation in efficiency, robustness, or the ability to measure multiple species.

More recently, He *et al* demonstrated separation of free polysaccharide from carrier protein and glycoconjugate using porous particle (300 Å pore size) reversed phase (RP) high-performance liquid chromatography (HPLC) operated in an isocratic flow format [10]. In this method the polysaccharides eluted before the void volume based on size. The free protein and conjugate were then eluted by running a gradient of increasing organic mobile phase. The separation space prior to the void volume in RPLC is typically ignored as most separations occur after the void where analytes are retained on a non-polar stationary phase and eluted based on relative

hydrophobicity. Polysaccharides are generally hydrophilic and have weak retention on non-polar stationary phases. The method conditions used by He *et al* had a mobile phase containing 20% organic, resulting in the polysaccharide being un-retained on a RPLC column. This eliminated interaction between the polysaccharide and the column, and with a porous column designed for macromolecules (300 Å pore size), a size-based separation was performed while retaining the other, more hydrophobic components in the sample mixture. This allowed for an additional separation space for molecules not typically compatible with RPLC. Though the focus of the method was on the resolution of the polysaccharide from the other components, the carrier protein peak was observed to elute after the glycoconjugate peak. The separation was not optimized for quantitation of the carrier protein as the method was intended for free polysaccharide analysis. Utilizing the principles demonstrated by He *et al*, it is believed that the use of a hydrophobic interaction chromatography (HIC) column could also resolve the free protein and conjugate in one run. The free polysaccharide would be separated by size prior to the void and the free protein would then be separated from the glycoconjugate using a non-denaturing aqueous mobile phase with an increasing gradient of ionic strength if needed. RP and HIC are similar in that separation is based on the interaction of hydrophobic patches on the surface of proteins with an immobilized non-polar ligand. However, RPLC stationary phases are more highly substituted than HIC stationary phases, resulting in much tighter binding of the adsorbents to the stationary phase and organic solvents are required for elution, which are denaturing to the proteins being analyzed. This could be problematic for hydrophobic proteins, such as CRM_{197}, and large glycoconjugates where highly retained adsorbent fail to elute. Using HIC with non-denaturing (native) separation conditions could also provide increased selectivity between the carrier protein and the glycoconjugate. After conjugation of the carrier protein to

the polysaccharide, the glycoconjugate should have differing surface hydrophobicity compared to the carrier protein. For these reasons, HIC will be explored as a potential mode of separation for free carrier protein and free polysaccharide.

1.3.HIC: A POTENTIAL MODE OF SEPARATION

Hydrophobic interaction chromatography (HIC) utilizes immobilized non-polar ligands to separate molecules based on hydrophobicity. The immobilized non-polar ligands interact with non-polar amino acids, such as phenylalanine, tryptophan, and valine, on the surface of proteins, termed "hydrophobic patches" [11]. Proteins have varying hydrophobic characteristics based on the number and sequence of non-polar amino acids that allows for separation by HIC. HIC is also termed "salt-promoted absorption" since the retention of the protein on the stationary phase is increased by mobile phases containing high concentrations of salts and are then eluted by running a gradient of decreasing salt concentration [12]. HIC is routinely utilized in protein purification steps during manufacturing, as well as purity analysis of recombinant proteins during drug product development and release testing. Suárez *et al* demonstrated separation of unbound *Streptococcus pnueumoniae* serotype 14 capsular polysaccharide (CPS14), unbound carrier protein, and CPS14-protein conjugate using a Sephadex CL-6B column at a preparative scale [13]. The separation was completely based on hydrophobic properties. The resolution of the peaks is less than ideal and can be potentially limiting if applied as a platform technology. Therefore, a HIC column was explored using the similar principles of separation as He *et al* but using mobile phases consisting of non-denaturing, low ionic strength rather than organic mobile phases utilized in RPLC.

SEC with a HIC column will be attempted as an alternative approach to MEKC and RPLC methods for quantitation of free carrier protein and polysaccharide in glycoconjugate vaccines. Utilizing a HIC column to separate by size could potentially offer several significant advantages for monitoring levels of free polysaccharide and free protein over existing technologies:

1. Improved reproducibility, precision, robustness, and throughput of an HPLC method over CE (MEKC) methods.
2. HIC utilizes more native conditions for separation compared to the denaturing environments associated with RPLC. This could avoid issues with highly retained adsorbents. Also, native separation conditions could also provide increased selectivity between the carrier protein and the conjugated carrier protein.
3. Both MEKC and RPLC methods require multiple methods for quantitation of free polysaccharide and protein. Whereas, with HIC, there is potential that quantitation of both free polysaccharide and free protein can be consolidated into a single method.

Furthermore, improving efficiency of the analytical support throughout the vaccine development process and release testing at commercial manufacturing sites conserves time, resources, and money, all of which equate to a lower cost for vaccine development and manufacturing, which is important for providing low cost vaccines to the developing world.

Hypotheses:

1. A separation based on size and relative hydrophobicity can be achieved using a HIC column to resolve polysaccharide, carrier protein, and glycoconjugate sufficiently to quantitate the amount of free carrier protein and free polysaccharide in glycoconjugate samples.

2. The developed technology can be successfully applied to vaccine development of similar glycoconjugate vaccine antigens utilizing similar protein carriers.

Goals:

1. Develop and optimize a HIC method with conditions to achieve acceptable separation and detection of carrier protein, polysaccharide, and glycoconjugate to quantitate free carrier protein and polysaccharide.
2. Perform an ICH qualification of the developed method to demonstrate method performance i.e. specificity, linearity, precision, accuracy, detection/quantitation limit, and range.
    a. Use results from ICH qualification to compare performance of developed HIC and current MEKC (in-house qualification data) methods
3. Explore feasibility of method as a platform method for various glycoconjugate serotypes utilizing the same carrier protein.

2. CHAPTER 2: 1D SEPARATION OF FREE CARRIER PROTEIN AND POLYSACCHARIDE BY HIC

This chapter describes the experiments performed for the development of a HIC method to separate and quantitate free carrier protein and polysaccharide in glycoconjugate vaccine samples. This method will be developed for two glycoconjugate drug substances, *S. aureus* capsular polysaccharide serotypes, CP5 and CP8 polysaccharide, conjugated to CRM_{197}. CP5 and CP8 polysaccharides serotypes have known structures published in literature and differ only by the linkages in the monosaccharides and sites of O-acetylation [14]. Though the two polysaccharides are similar in structure, they differ serologically.

2.1. INSTRUMENTS

Chromatographic experiments in this work were performed on Agilent 1100 and 1200 HPLC systems (Santa Clara, CA) equipped with a binary/quaternary pump, autosampler, column compartment, diode array detector (DAD)/variable wavelength detector (VWD), and for column-screening experiments, a 6-port/2 position valve was utilized. An Empower 3 data acquisition system was used for controlling the instruments, data acquisition, and analyzing data. However, when using the 6-port/2-position switching valve, Chemstation was used utilized for controlling the instrument, data acquisition, and analyzing data.

2.2. SAMPLES

Purified reference standards of the individual components were used during the method development in additional to glycoconjugate samples. Individual components were injected to confirm and track the free components in the spike sample. Concentrations of protein and polysaccharide were obtained by colorimetric techniques. The protein concentration was

determined using the modified Lowry method, where the sample is reacted under alkaline conditions with cupric sulfate and tartrate ion to produce a tetradentate protein-copper complex [15]. A Folin-Ciocalteu reagent is then added and effectively reduced by the protein-copper complex resulting in a blue color proportional to the protein-copper complex concentration and can be measured at 750 nm. The saccharide concentration was determined by the PAHBAH (p-hydroxybenzoic acid hydrazide) assay, where the saccharide sample is hydrolyzed to individual monosaccharides using hydrochloric acid and heat [16]. The monosaccharides are then reacted with the PAHBAH reagent to yield yellows anions proportional to the monosaccharide concentration that can be measured at 410 nm. Samples were transferred to HPLC vials and directly injected for analysis. The samples used are listed in Table 1.

Table 1 Sample Information for 1D Chromatography Development

Name	Concentration (mg/mL)	Preparation
Placebo (L-Histidine)	n/a	n/a
CP5 Polysaccharide Reference Standard	1.0	Diluted to 0.1 mg/mL in water.
CP8 Polysaccharide Reference Standard	1.0	Diluted to 0.1 mg/mL in water.
Carrier Protein Reference Standard	5.4	Diluted to 0.1 mg/mL in water.
CP5-CRM_{197} Glycoconjugate	1.8 (1:1 ratio of polysaccharide: protein)	No preparation.
CP8-CRM_{197} Glycoconjugate	1.8 (1:1 ratio of polysaccharide: protein)	No preparation.

The method development typically focused on one serotype for the majority experiments. The purpose was to increase efficiency as the structures of the two serotypes are similar. Therefore, the physical/chemical characteristics exploited for separation were not expected to differ greatly between the serotypes.

2.3.HIC METHOD DEVELOPMENT

The following sections describes the experiments for developing a HIC method for analyzing free carrier protein and polysaccharide.

2.3.1. COLUMN SCREENING – VENDOR, STATIONARY PHASE, AND PORE SIZE

Several HIC columns were evaluated to potentially identify an effective column for this application. HIC columns have various attributes that can affect a separation, and screening can help narrow down the critical attributes that have significant impact. The columns chosen to evaluate are listed in Table 2. As a size-based separation for the polysaccharide prior to the void was intended, a porous column was desired. Therefore, a range of pore sizes were evaluated. A non-porous column (TSKgel Butyl-NPR) was also tested to help confirm the impact of porosity on the separation by size prior to the void. Also, substituted functional groups with a range of hydrophobicity were selected to probe the impact of on the interaction with the hydrophobic carrier protein and glycoconjugate.

Table 2 HIC Columns Evaluated

Column Name	Vendor	Material Functional Group	Particle Size (µm)	Pore size (Å)	Dimension (mm)
ProPac® HIC-10	Thermo Scientific	Ethyl	5.0	300	7.8 x 75
TSKgel Phenyl-5PW	TOSOH	Phenyl	10 - 30	1,000	7.5 x 75
Shodex HIC PH-814	Shodex	Phenyl	8.0	2,000	8.0 x 75
TSKgel Butyl-NPR	TOSOH	Butyl	2.5	Non-porous	4.6 x 35

The mobile phase used for this screening experiment was 50 mM sodium phosphate, pH 7.0 with an isocratic flow rate of 0.8 mL/min. A neutral, low salt mobile phase was used to minimize interaction with the column. The salt concentration used is much lower than typically seen in a HIC separation for macromolecules, where salt concentrations can range from 0.5 to 2.0 M. However, for this application of performing a sized-base separation prior to the void volume on a HIC column, minimal interactions with the column are desired while still providing adequate resolution of the peaks of interest. The columns were held at ambient temperature (approximately 22° C) during analysis. UV detection at 210 nm was used for detection of the peaks of interest. The samples listed in Table 1 were individually injected at 12 to 20 µL to

target approximately 2 µg on the column. An overlay of the individual components analyzed by each HIC column is shown in Figure 2 through Figure 5. For the analysis, the chromatographic profile was qualitatively assessed for resolution of the individual components prior to the inclusion void for each of the columns evaluated. Ideally, a column would be identified that provides the maximum resolution between the three main components, carrier protein, polysaccharide, and glycoconjugate. The component of particular focus was the polysaccharide. As the polysaccharide is hydrophilic in nature and expected to have little to no interaction with hydrophobic stationary phase, the separation conditions must provide a suitable peak profile for analysis. Note, glycoconjugate samples are formulated in a histidine buffer. Therefore, the carrier protein was diluted in histidine for this experiment. In addition, histidine was analyzed as a blank to identify the matrix-related peak and the inclusion volume of the column. The polysaccharide samples are formulated in water, and no matrix-related peaks is expected.

The overlay of the analysis using the ProPac HIC-10 manufactured Thermo Scientific is shown in Figure 2. All of the injected components were observed and resolved from the histidine matrix peak (inclusion void). In addition, all peaks showed acceptable peak shape, even for the larger, heterogeneous polysaccharide and glycoconjugates. The carrier protein was resolved from the other components, however, the glycoconjugate and polysaccharide peaks were observed to co-elute. These observations are similar to separations using SEC where the smaller molecule carrier protein is resolved, but the polysaccharide and glycoconjugate co-elute due to similar hydrodynamic radii. This data shows potential as a suitable HIC column for the intended application of free carrier protein and polysaccharide.

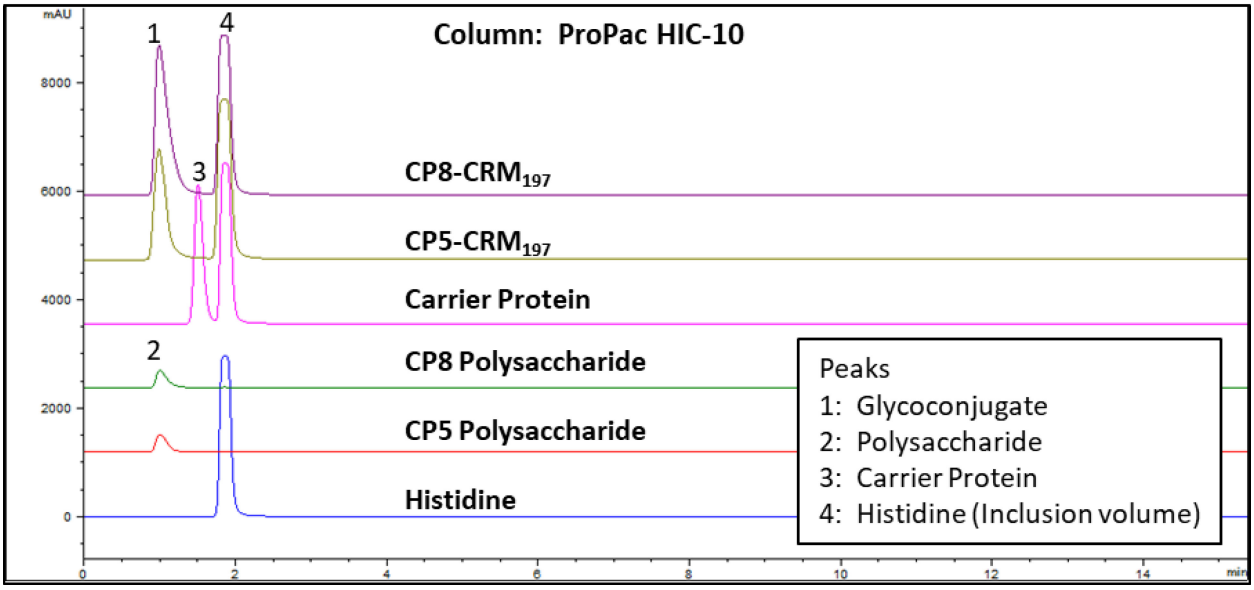

Figure 2 HIC analysis of CP5 and CP8 glycoconjugate samples, carrier protein, CP5 and CP8 polysaccharides, and histidine matrix using ProPac HIC-10 column. All components were observed eluting prior to void and separated from the histidine matrix.

The overlay of the analysis using the TSKgel Phenyl-5PW manufactured by Tosoh is shown in Figure 3. The glycoconjugate peaks were not observed, which could be due to retention on the column or co-elution with the histidine matrix peak. The carrier protein (approximately 58 kDa) was observed to elute later near the histidine matrix peak (inclusion void), likely due to the carrier protein being on the lower end of the molecular weight range of a column with a 1,000 Å pore size. For example, the Agilent Bio SEC-5 column with a 1000 Å pore size has a molecular weight range of 50 – 7,500 kDa. Also, the polysaccharide peak was observed to be very broad with poor peak shape. The current separation conditions with this column do not provide acceptable size-base separation prior to the void. However, this column could show potential with further development as the glycoconjugates were likely retained, which could be used for selectively between the polysaccharide and other hydrophobic components.

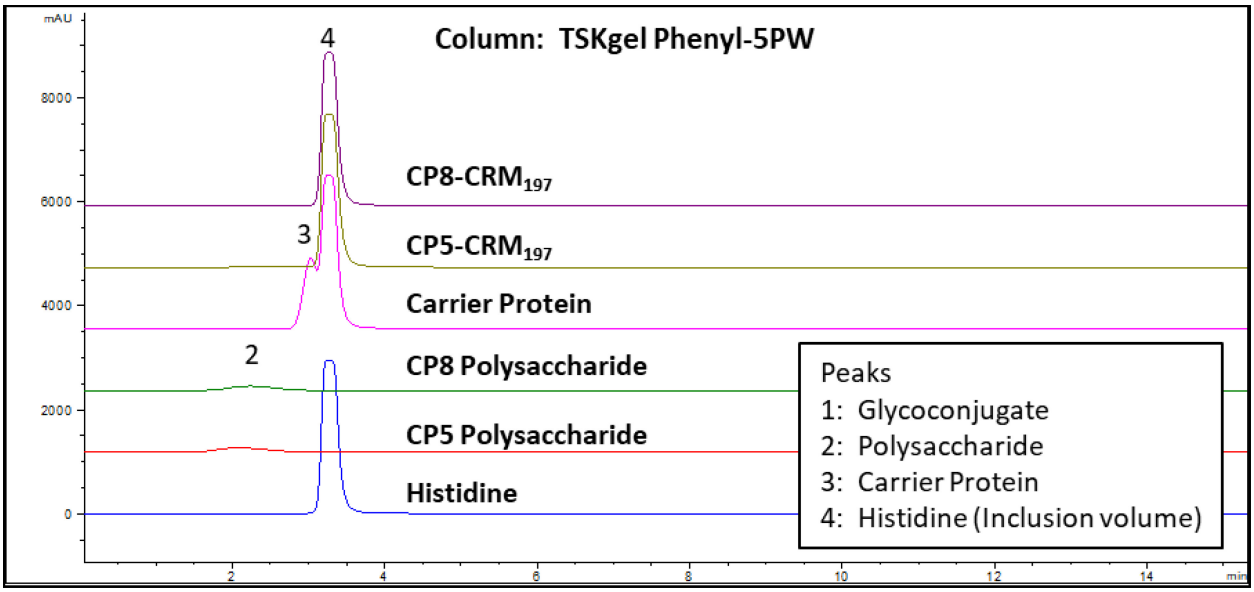

Figure 3 HIC analysis of CP5 and CP8 glycoconjugate samples, carrier protein, CP5 and CP8 polysaccharides, and histidine matrix using TSKgel Phenyl-5PW column. Glycoconjugate peaks were not observed and possibly retained. Carrier protein peak co-eluted with histidine matrix peak. Broad polysaccharide peaks were observed.

The overlay of the analysis using the Shodex HIC PH-814 is shown in Figure 4. All of the injected components were observed. Similar to the TSKgel Phenyl-5PW column, the carrier protein was observed co-eluting with the histidine matrix peak, likely due to the same reasons of being on the lower end of the molecular weight calibration range of the column. The polysaccharides were observed to be broad with poor peak shape. The glycoconjugate peaks were observed, unlike the TSKgel Phenyl-5PW, which both utilize a phenyl substituted stationary phase. Therefore, retention of the glycoconjugate might have been expected for both columns. This could indicate interactions other than hydrophobicity are driving the retention under these conditions for the TSKgel Phenyl-5PW column. The cause of this would require further investigation with follow-up experiments. In general, all peaks of interest were observed to be broad, indicating potential secondary interactions with the particle. The current separation

conditions using the Shodex HIC PH-814 do not provide acceptable size-base separation prior to the void.

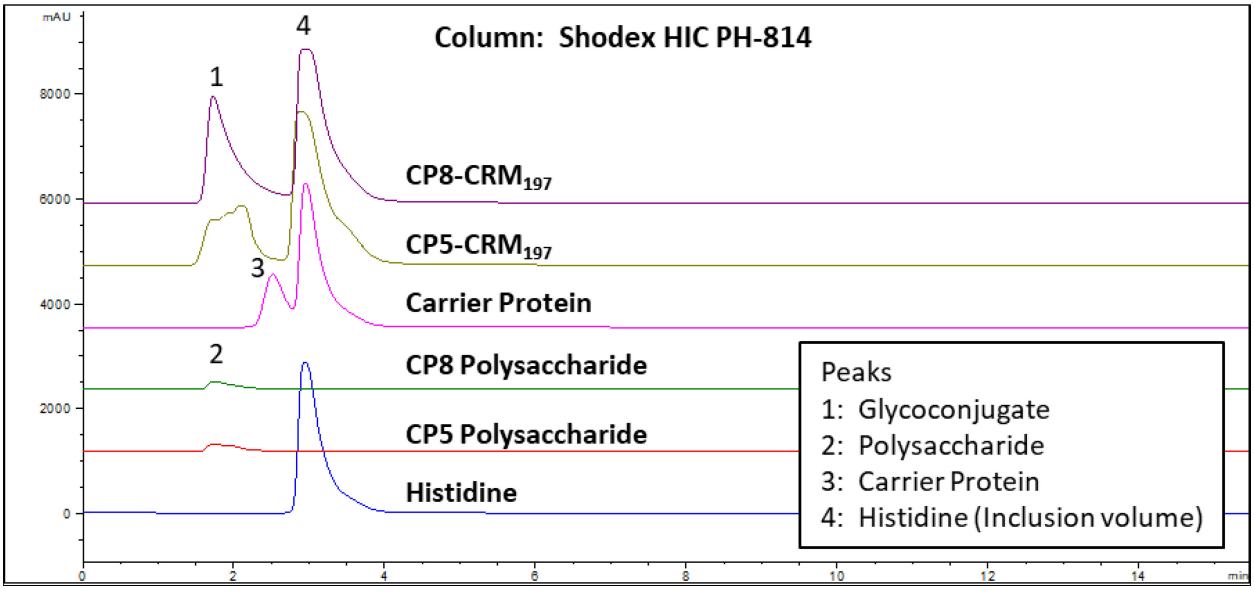

Figure 4 HIC analysis of CP5 and CP8 glycoconjugate samples, carrier protein, CP5 and CP8 polysaccharides, and histidine matrix using Shodex HIC PH-814 column. All components were observed eluting prior to void. The carrier protein peak observed to slightly co-elute with the histidine matrix peak. Broad polysaccharide peaks observed.

Lastly, the overlay of the analysis using the TSKgel Butyl-NPR manufactured by Tosoh is shown in Figure 5. As expected with a non-porous HIC column with low ionic strength mobile phase, all components were un-retained and eluted near the void volume. Since the hydrophilic polysaccharide is un-retained and the column is non-porous, there are no physical or chemical properties of the polysaccharide to provide selectivity from the other components. Therefore, the analysis of free polysaccharide does not appear feasible on this column.

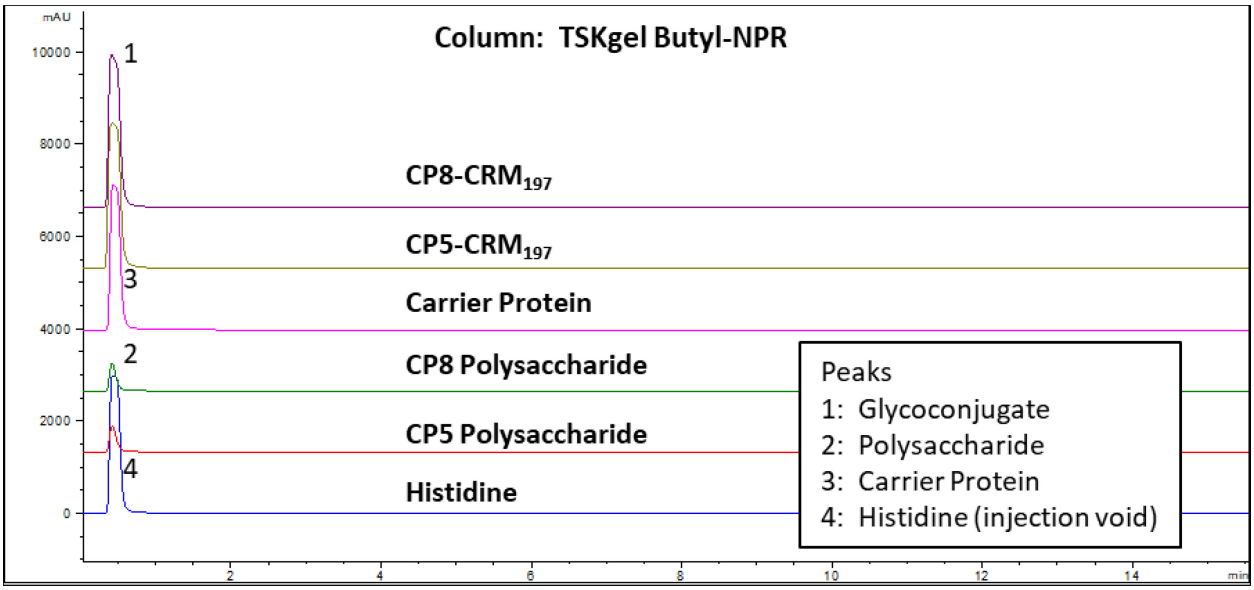

Figure 5 HIC analysis of CP5 and CP8 glycoconjugate samples, carrier protein, CP5 and CP8 polysaccharides, and histidine matrix using TSKgel Butyl-NPR column. All components were observed eluting near the void volume as the non-porous column does not provide separation by size.

In summary, the Thermo HIC-10 column provided the greatest resolution between the individual components analyzed along with suitable peak profiles for the polysaccharide and carrier protein. The Tosoh column showed potential in being effective if operated under normal HIC separation conditions (high salt) where the analytes (carrier protein and glycoconjugate) are retained and eluted after the void volume. However, further development would be needed to optimize the polysaccharide peak shape and elution condition of the glycoconjugate. Therefore, the Thermo HIC-10 column was further used in development experiments for the application of free carrier protein and polysaccharide analysis using HIC. Similar to the SEC separation, the polysaccharide and glycoconjugate peaks were shown to co-elute, therefore, in order to modify the selectivity between the glycoconjugate and the polysaccharide peaks, slight modifications to the separation conditions, such as pH and salt concentration, which are key parameters that affect retention in a HIC, were first explored.

2.3.1. MOBILE PHASE PH

The impact of the mobile phase pH was first evaluated as this parameter is useful in modifying selectivity in a HIC separation. The pH impacts the ionization state of a protein, which is related to the hydrophobicity of the protein. So, altering the pH of the mobile phase could potentially be used for modifying selectivity between the protein-related components and the polysaccharide. Also, evaluating the pH can provide robustness information for these separation conditions. The initial column screening experiment was performed with a mobile phase 50 mM sodium phosphate, pH 7.0. Therefore, mobile phases were prepared to a final pH ranging from 6.0 – 7.5, most of the sodium phosphate buffering range. The Thermo HIC-10 column was used with an isocratic flow rate of 1.0 mL/min, 25° C column compartment, and UV detection at 210. The carrier protein and CP8 polysaccharide listed in Table 1 were individually injected at 10 µL to target approximately 1 µg on the column. An overlay of the carrier protein and CP8 polysaccharide analyzed at each pH is shown in Figure 6 and Figure 7, respectively. The isoelectric point (pI) is the pH value at which the overall charge state of a protein is neutral. The pI of the carrier protein (CRM_{197}) was determined to be 6.21 using EMBOSS calculation software [17]. As the pH increased, the retention time of the carrier protein decreased. This can be explained by a decrease in hydrophobicity as the pH value moves away from the pI making the overall charge state of the carrier protein more negative. Retention of the carrier protein was reduced with each incremental increase in pH. At pH values of 7.0 and lower, the carrier protein peak began to elute later and closer to the void volume resulting in co-elution with the histidine peak, which was shown to elute in the void volume. For a separation of carrier protein prior to the void volume under these conditions, a pH of 7.0 and lower would not be suitable. The CP8 polysaccharide peak eluted in front of the void volume and remained unchanged with respect to

retention time, peak profile, and peak height at all pH values tested. This showed pH of the mobile phase did not have an impact on the polysaccharide peak across this pH range.

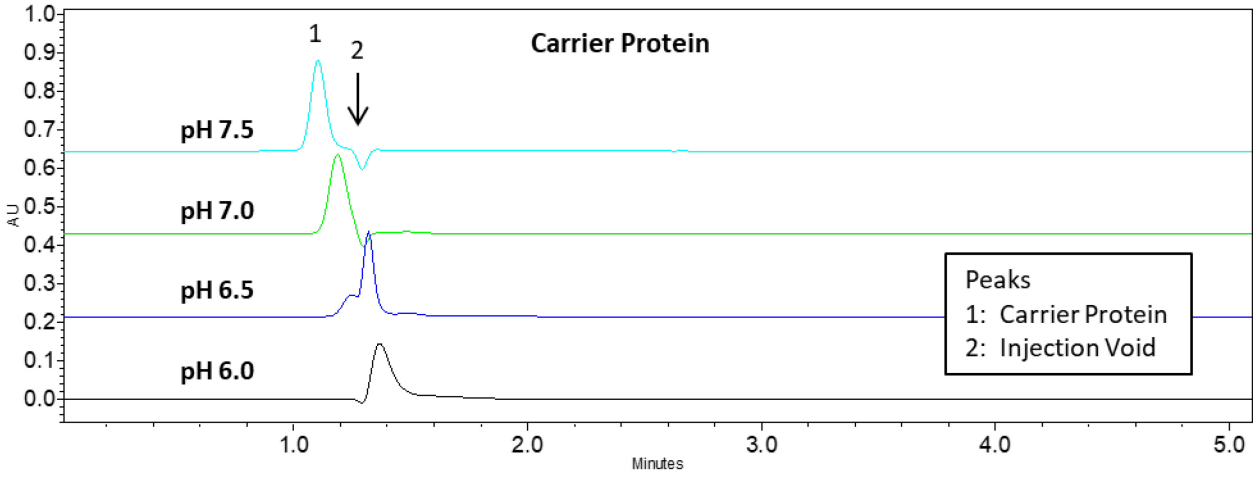

Figure 6 HIC analysis of carrier protein using a Thermo HIC-10 column with 50 mM sodium phosphate prepared at pH values 6.0, 6.5, 7.0, and 7.5. An increase in retention time was observed as pH decreased.

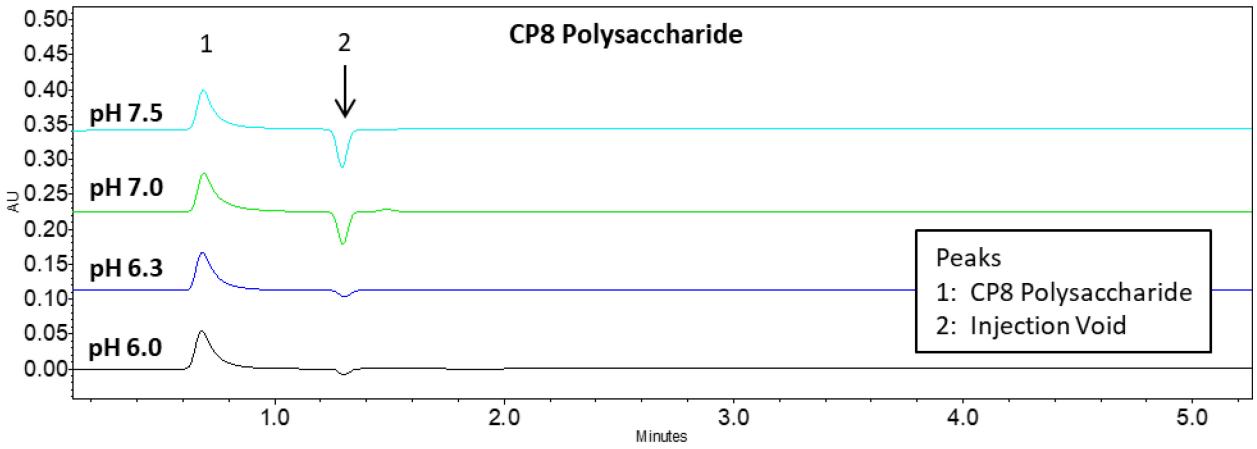

Figure 7 HIC analysis of CP8 polysaccharide using a Thermo HIC-10 column with 50 mM sodium phosphate prepared at pH values at 6.0, 6.3, 7.0, and 7.5. No impact was observed on the polysaccharide peak across the pH values tested.

Overall, a higher pH, 7.0 to 7.5, appeared to yield acceptable peak profiles and resolution of both the polysaccharide and carrier protein peaks for a sized-base separation prior to the void volume. A higher pH will be utilized in further experiments with this strategy in mind.

2.3.2. MOBILE PHASE BUFFER CONCENTRATION

The salt concentration of the mobile phase was evaluated next using the Thermo HIC-10 column. Increasing the salt concentration in the mobile phase promotes the salting-out effect in a HIC separation resulting in increased retention. As the carrier protein eluted near the void volume in the previous experiment, slight modifications to the salt concentration could potentially achieve better resolution, and in addition, provide information on the impact of minimal changes to mobile phase concentration. Mobile phases were prepared ranging from 10 to 100 mM sodium phosphate at a pH of 7.5. An isocratic flow rate of 1.0 mL/min with UV detection at 210 was used. The column was held at 25° C. Carrier protein and CP8 polysaccharide listed in Table 1 were individually injected at 10 µL to target approximately 1 µg on the column. An overlay of the carrier protein and CP8 polysaccharide analyzed at each pH is shown in Figure 8 and Figure 9, respectively. As expected, as the salt concentration increased, the retention time of the carrier protein increased. The retention time of the polysaccharide did not shift with increasing concentration as the carrier protein did. However, at a concentration of 75 mM, the polysaccharide peak began to broaden with a decrease in peak height and area count. This could be due to the salt inducing non-specific interactions with the column stationary phase or slow desorption kinetics leading to band broadening.

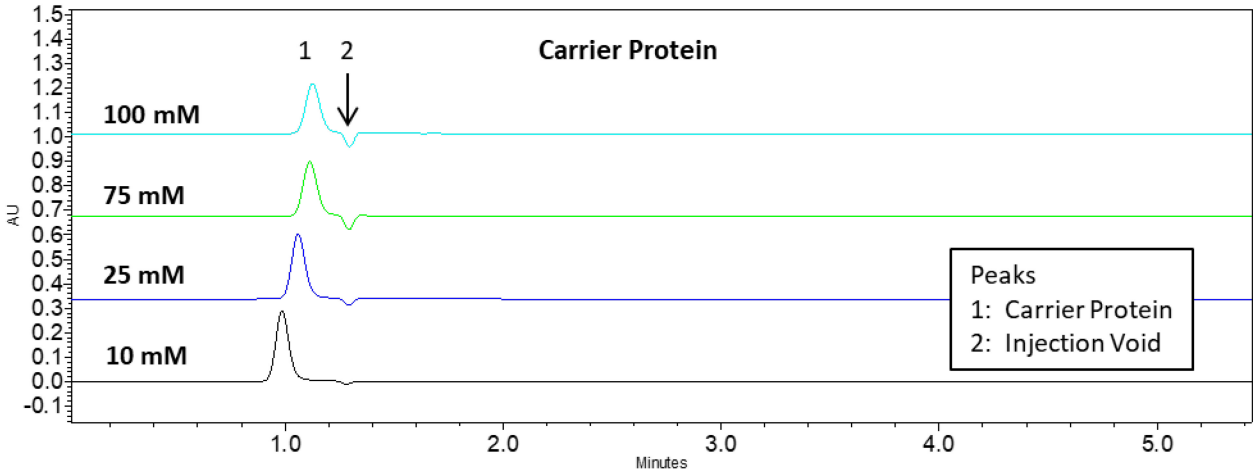

Figure 8 HIC analysis of carrier protein using a Thermo HIC-10 column with sodium phosphate, pH 7.5 prepared at concentrations of 10, 25, 75, and 100 mM. An increase in retention time was observed as salt concentration increased.

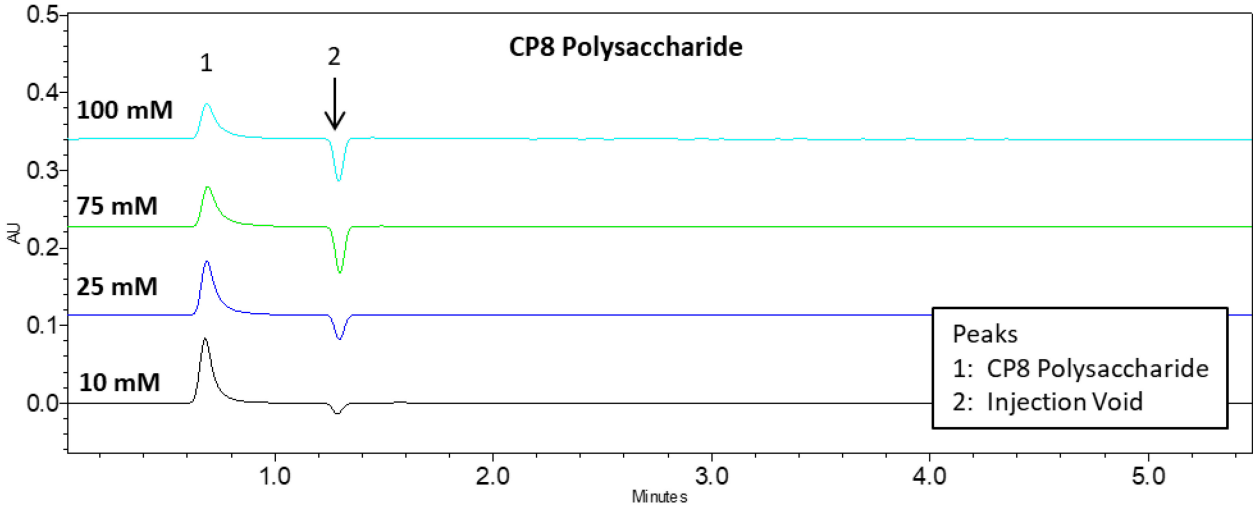

Figure 9 HIC analysis of CP8 polysaccharide using a Thermo HIC-10 column with sodium phosphate, pH 7.5 prepared at concentrations of 10, 25, 75, and 100 mM. The peak broadened and decreased in height as salt concentration increased.

The lowest salt concentration of 10 mM showed the best results for both the carrier protein and the polysaccharide peaks suggesting mainly a sized-based separation. The carrier protein was most resolved from the void volume while still being baseline separated from the polysaccharide. Also, the polysaccharide peak showed improved peak shape and the highest recovery in terms of area count. A salt concentration of 10 mM will be utilized for the following experiments with the Thermo HIC-10 column.

2.3.3. GLYCOCONJUGATE EVALUATION ON THERMO HIC-10 COLUMN

The mobile phase conditions were optimized for separation of the carrier protein and polysaccharide in the previous experiments using the Thermo HIC-10 column. The next step was applying the conditions to the analysis of a glycoconjugate sample and evaluate its behavior. A glycoconjugate is a composition of carrier protein and polysaccharide, and therefore structurally different than the individual carrier protein and polysaccharide molecules. These structural differences were thought to potentially provide selectivity between the individual components in a HIC separation. The Thermo HIC-10 column was used with an isocratic flow rate of 1.0 mL/min and UV detection at 210. The mobile phase was 10 mM sodium phosphate, pH 7.5. The column was held at 25° C. The carrier protein, CP8 polysaccharide, and CP8-CRM_{197} glycoconjugate listed in Table 1 were individually injected to target approximately 1 µg on the column. An overlay of the carrier protein, CP8 polysaccharide, and CP8-CRM_{197} glycoconjugate injections are shown in Figure 10. The polysaccharide and glycoconjugate peaks were observed to co-elute, but resolved from the carrier protein, similarly to the analysis by SEC. This indicates that the properties of the polysaccharide in the glycoconjugate are likely driving the retention behavior under these native separation conditions. This data further supports the separation prior to void is size-based with minimal interaction with the stationary phase. However, this also shows these separation conditions are not suitable for the analysis of free polysaccharide. To resolve the glycoconjugate and polysaccharide peaks, additional selectivity would be needed. Therefore, HIC parameters would be re-evaluated, and new parameters explored with the addition of the glycoconjugate sample.

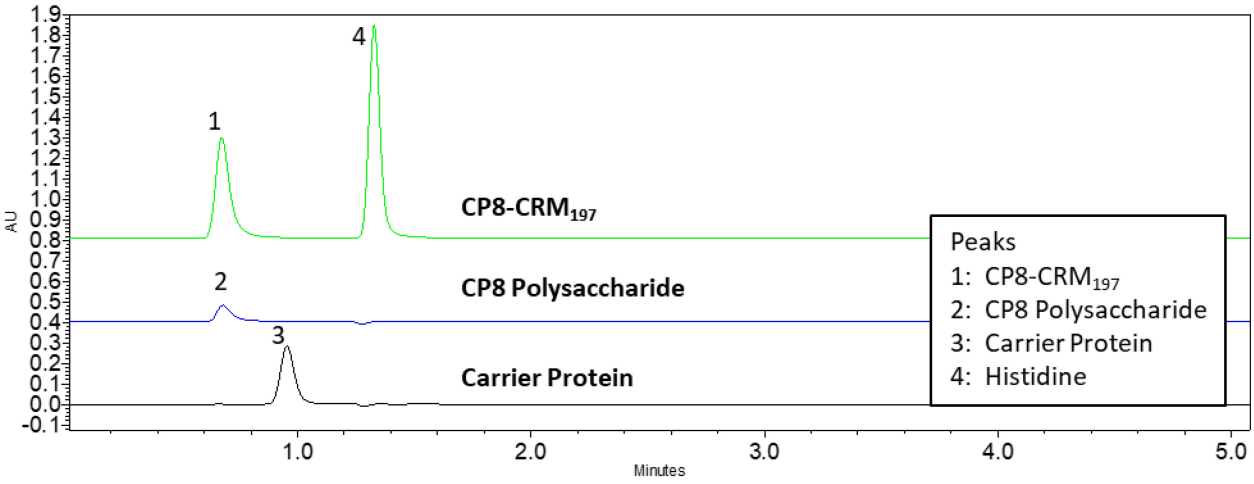

Figure 10 HIC analysis of carrier protein, CP8 polysaccharide, and CP8-CRM$_{197}$ glycoconjugate using a Thermo HIC-10 column with 10 mM sodium phosphate, pH 7.5. Co-elution of the polysaccharide and glycoconjugate peaks was observed.

The salt concentration and pH of the mobile phase were both shown to increase retention of the carrier protein. These parameters were both further evaluated with the analysis of a glycoconjugate sample. To increase retention of the glycoconjugate, the mobile phase pH was decreased from 7.5 to 6.0, and the sodium phosphate concentration was increased from 10 to 50 mM. The goal was to induce subtle interactions between the glycoconjugate and stationary phase without disrupting the polysaccharide peak. All other separation parameters were the same as the previous experiment. An overlay of the carrier protein, CP8 polysaccharide, and CP8-CRM$_{197}$ glycoconjugate injections are shown in Figure 11. Again, the polysaccharide and glycoconjugate peaks were observed to co-elute. The glycoconjugate peak did slightly shift and appear broader than the previous method conditions with higher pH and lower salt concentration. This further indicates that under these separation conditions, the retention behavior of the glycoconjugate is primarily influenced by the polysaccharide.

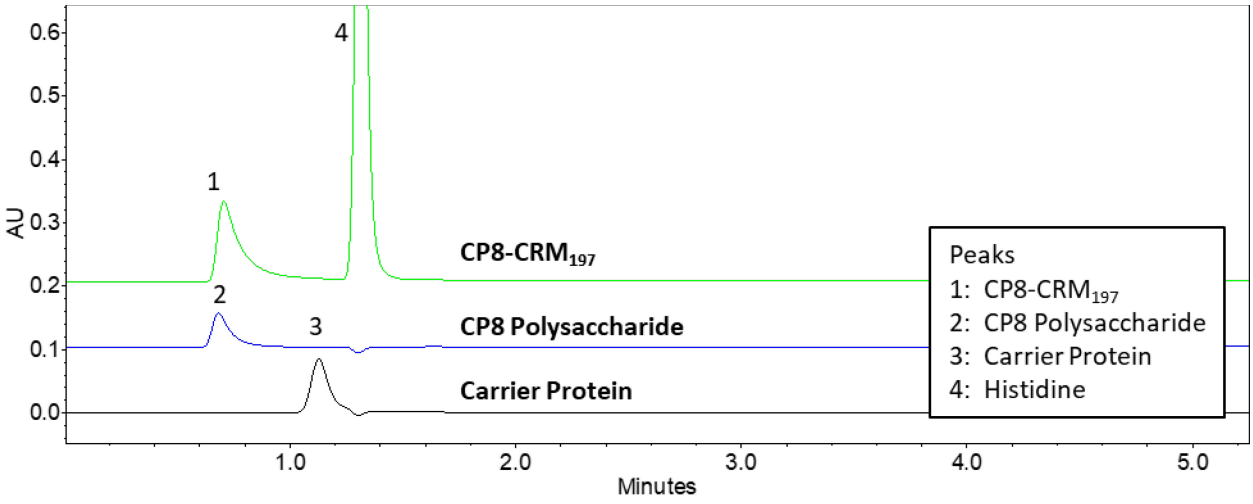

Figure 11 HIC analysis of carrier protein, CP8 polysaccharide, and CP8-CRM197 glycoconjugate using a Thermo HIC-10 column with 50 mM sodium phosphate, pH 6.0. Co-elution of the polysaccharide and glycoconjugate peaks was still observed with lower pH and slightly

Overall, the subtle changes in mobile phase pH and salt concentration failed to provide additional selectivity to resolve the glycoconjugate and polysaccharide.

2.3.4. Salt Gradient using Thermo HIC-10

Since the polysaccharide and glycoconjugate peaks were shown to co-elute with the previously developed conditions, further modifications of separation parameters were needed, specifically in increasing retention of the glycoconjugate. Traditional HIC separation utilized a descending gradient from high to low salt concentration. Sodium sulfate salt was chosen for this evaluation as its better able to salt out proteins compared to sodium phosphate according to the Hoffmeister series, which classifies ions for their ability to salt out proteins based on their binding strength at a given concentration. With this approach, the protein-related peaks would salt out in the strong solvent inducing retention on the stationary phase while the polysaccharide would be un-retained and elute prior to the void volume. A descending gradient of salt would then elute the carrier protein and glycoconjugate. Mobile phase A (weak solvent) was of 0.75 M sodium sulfate in 0.05 M sodium phosphate, pH 6.3, and mobile phase B (strong solvent) was 0.05 M sodium

phosphate, pH 6.3. The Thermo HIC-10 column was used with a flow rate of 1.0 mL/min and UV detection at 210. The following gradient was run: hold at 0% B for 10 min., increase to 50% B at ramp to 80% B over 15 min., increase to 95% B and hold 5 min., return to 0% B and hold for 10 min. The column was held at 25° C. The carrier protein, CP8 polysaccharide, and CP8-CRM197 glycoconjugate listed in Table 1 were individually injected to target approximately 1 µg on the column. An overlay of the carrier protein, CP8 polysaccharide, and CP8-CRM197 glycoconjugate injections are shown in Figure 12. The polysaccharide and carrier protein peaks were observed to elute, but both showed poor peak profiles. The polysaccharide co-eluted with the void volume, while the carrier protein was more retained with the higher ionic strength mobile phase and eluted after the void volume. The glycoconjugate peak was not observed however. The method gradient reduces the salt concentrations in the mobile phase near concentrations previously shown to elute the glycoconjugate, and elution was expected. However, the lack of the glycoconjugate peak could indicating strong hydrophobic interactions with the stationary phase potentially leading to irreversible binding.

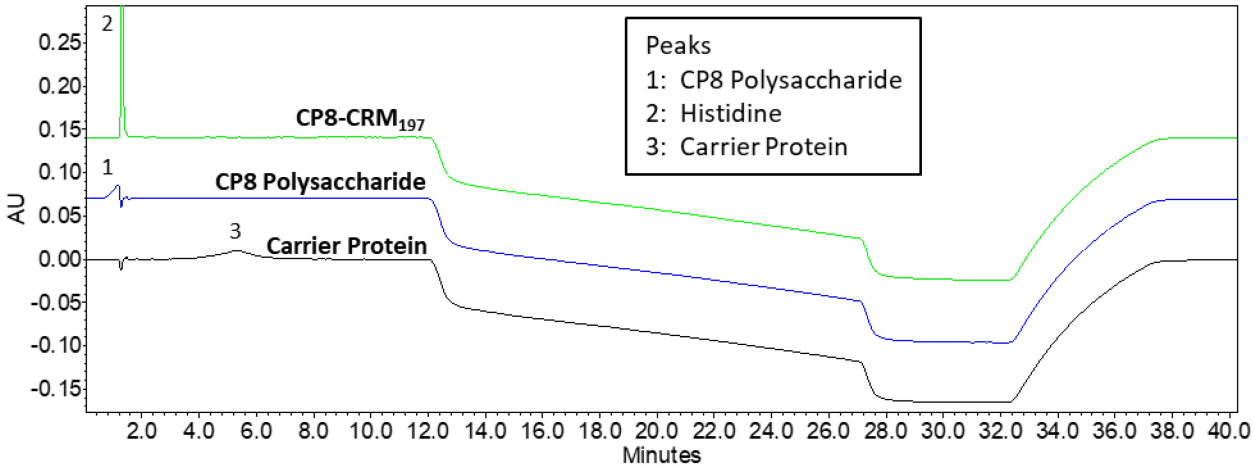

Figure 12 HIC analysis of carrier protein, CP8 polysaccharide, and CP8-CRM$_{197}$ glycoconjugate using a Thermo HIC-10 column with 0.75 mM sodium sulfate gradient in 50 mM sodium phosphate, pH 6.3. CP8-CRM$_{197}$ not observed likely due to strong retention. Polysaccharide peak observed prior to the void volume with poor peak shape. Carrier protein peak was observed to be broad and eluted after the void volume.

Several important observations were made with the results from this experiment. The first being the glycoconjugate could be irreversibly bound to the stationary phase using higher ionic strength mobile phase. This indicates stronger elution conditions are likely required to disrupt the tight binding to the stationary phase, potentially with the use of chaotropic modifiers such as isopropanol. Secondly, compared to the glycoconjugate, the carrier protein did not exhibit the same tight binding, showing the behavior of the carrier protein differs from the glycoconjugate under these conditions. Thirdly, the polysaccharide peak profile deteriorated with higher ionic strength mobile phase e.g. fronting, loss of peak height, and co-elution with void volume. This is critical as the strategy for retaining the protein-related peaks with higher ionic strength mobiles phases may not yield an acceptable profile for the polysaccharide peak. In summary, these HIC separations conditions are not suitable for the quantitation of free carrier protein and polysaccharide, and further development would be needed to overcome the issues observed.

2.3.5. SALT GRADIENT USING TOSOH TSKGEL PHENYL

Several approaches were taken for separating free carrier protein and polysaccharide from the glycoconjugate using the Thermo HIC-10 column with little success. So, an alternative column from previous experiments was re-evaluated. The Tosoh TSKgel Phenyl-5PW showed retention of the glycoconjugate under lower salt conditions (50 mM sodium phosphate) in the column screening experiment (section 2.3.1). The different stationary phase might provide reversible retention of the glycoconjugate. In addition, the lower ionic strength mobile phase with a slightly higher flow rate could yield a better peak profile for the polysaccharide. A descending gradient of salt was used to elute the glycoconjugate. Mobile phase A was 50 mM sodium phosphate, pH 7.0, and mobile phase B was 10 mM sodium phosphate, pH 7.0. A flow rate of 0.8 mL/min. was used with UV detection at 210 nm. The column was at ambient temperature. The following gradient was run: hold at 0% B for 10 min., 0 – 100% B over 15 min., hold at 100% B for 5 min., return to 0% B and hold for 5 min. The carrier protein, CP8 polysaccharide, and CP8-CRM_{197} glycoconjugate listed in Table 1 were individually injected to target approximately 1 µg on the column. Histidine was also injected as a blank. An overlay of the histidine, carrier protein, CP8 polysaccharide, and CP8-CRM_{197} glycoconjugate injections are shown in Figure 13. The polysaccharide and carrier protein peaks were observed to elute prior to the void volume. The polysaccharide showed better peak shape compared to the initial column screening experiment. The carrier protein was shown to co-elute with the histidine peak at the void volume. The glycoconjugate failed to elute with the descending salt gradient, showing the Tosoh TSKgel Phenyl-5PW exhibited the same tight binding observed with the Thermo HIC-10 column. In all injected samples, inconsistent responses were observed in the elution gradient at

approximately 15 and 20 minutes. This could be carry-over from previous injections of the glycoconjugate sample and would be further probed.

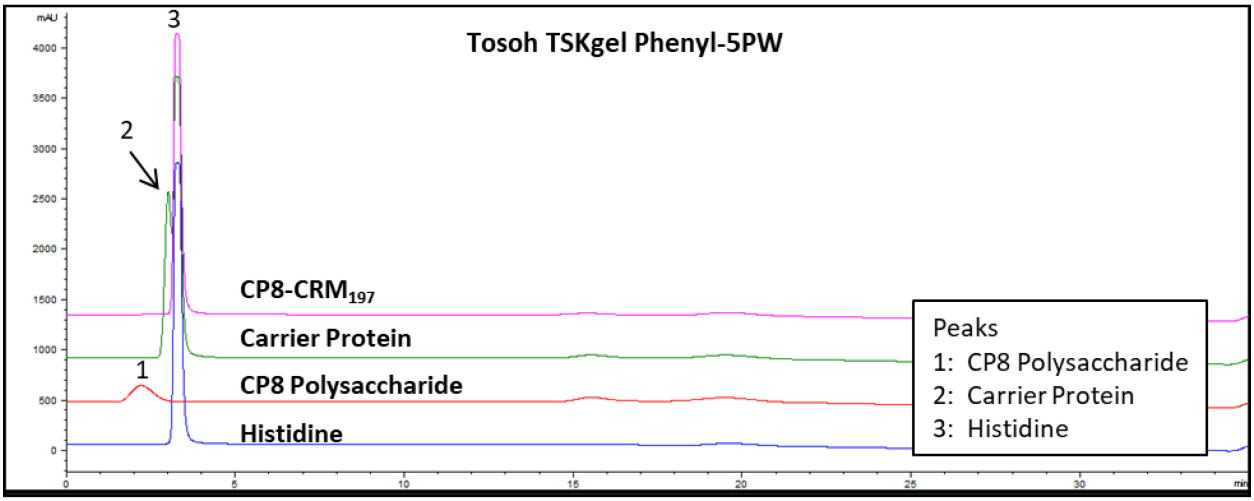

Figure 13 HIC analysis of the histidine matrix, carrier protein, CP8 polysaccharide, and CP8-CRM$_{197}$ glycoconjugate using a Tosoh TSKgel Phenyl-5PW column with gradient from 50 – 10 mM sodium phosphate, pH 7.0. CP8-CRM$_{197}$ failed to elute under low ionic strength conditions. CP8 polysaccharide and carrier protein were observed prior to the void volume. Responses were observed in gradient at approximately 15 and 20 min., potentially due to partial glycoconjugate elution from previous injections.

The column manufacturer recommends using sodium hydroxide (NaOH) regularly as a cleaning solvent to solubilize and remove tightly bound proteins. To investigate the observed response in the elution gradient and the lack of glycoconjugate peak in the previous experiment, a sequence was programmed the following day beginning using the same method conditions. The sequence contained a series of injections of 0.1N NaOH at 100 µL, followed by an injection of CP5-CRM$_{197}$, and bracketed by another injection of 0.1N NaOH at 100 µL. These injections are shown in an overlay along with the CP5-CRM$_{197}$ injection from sequence on the day prior in Figure 14. The first injection of NaOH shows a large response with two distinct peaks in the elution gradient at similar retention times previously observed. The following injection of NaOH does not have the same large response and the peak at 15 minutes is not visible. The broad peak is still observed at 20 minutes in all subsequent injections, which could be due to the elution

conditions not being strong enough to fully elute the glycoconjugate. Also, since the large peak is not observed in the NaOH injection following the glycoconjugate injection, this could indicate the glycoconjugate is building up on the column over repeated injections. The data supports the possibility that the glycoconjugate could be irreversibly bound to the column stationary phase.

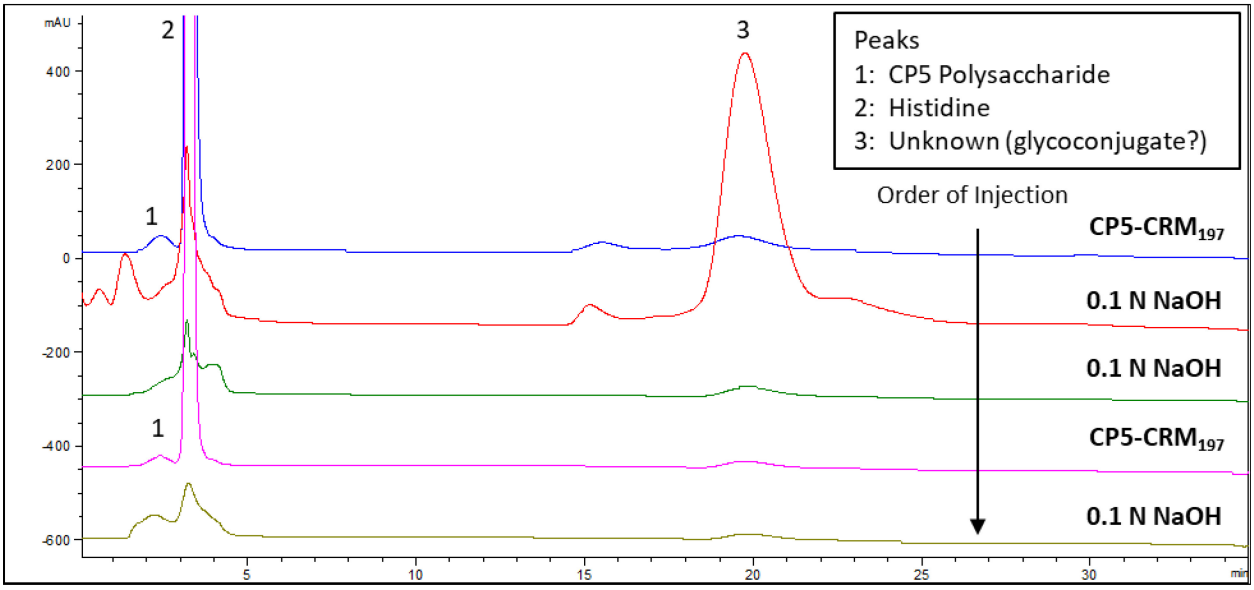

Figure 14 HIC analysis of the NaOH and CP5-CRM$_{197}$ glycoconjugate using a Tosoh TSKgel Phenyl-5PW column with gradient from 50 – 10 mM sodium phosphate, pH 7.0. CP8-CRM$_{197}$ failed to elute using low ionic strength mobile phase.

The method conditions evaluated using the Tosoh TSKgel are not acceptable as resolution of the carrier protein and polysaccharide is not achieved, and the elution conditions are not capable of eluting the tightly bound glycoconjugate.

2.3.6. High Ionic Strength with Chaotropic Modifier

In the previous section, a low ionic strength mobile phase was used with a descending gradient of salt. That yielded unacceptable resolution of the carrier protein and polysaccharide peaks. In addition, the glycoconjugate failed to elute and appeared to build up on the column as NaOH cleaning injections showed several large, additional peaks. HIC has been routinely applied to

macromolecules, such as antibody drug conjugates (ADCs) for drug-to-antibody (DAR) analysis [18]. These separations utilize high ionic strength salts in the strong solvent along with chaotropic modifiers (organic e.g. isopropanol) in the elution buffer to help elute hydrophobic proteins. This strategy was applied to the glycoconjugate sample for free carrier protein and polysaccharide analysis. This could potentially increase selectivity for the carrier protein and resolve it from the polysaccharide peak and void volume. Also, the chaotropic modifier could help disrupt interaction with the stationary phase and help elute the glycoconjugate. Mobile phase A was 0.75 M ammonium sulfate in 50 mM potassium phosphate, pH 7.0, and mobile phase B was 50 mM potassium phosphate, pH 7.0, with 10% Isopropanol. A flow rate of 0.8 mL/min. was used with UV detection at 210 nm. The column was at ambient temperature. The following gradient was run: hold at 0% B for 5 min., 0 – 95% B over 38 min., hold at 95% B for 5 min., return to 0% B and hold for 7 min. The carrier protein, CP8 polysaccharide, and CP8-CRM_{197} glycoconjugate listed in Table 1 were individually injected to target approximately 1 µg on the column. An overlay of the carrier protein, CP8 polysaccharide, and CP8-CRM_{197} glycoconjugate injections are shown in Figure 15. The polysaccharide was observed to elute prior to the void volume, but with a more acceptable peak profile compared to other experiments with higher ionic strength mobile phase. The carrier protein and the glycoconjugate peaks were not observed and appeared to be irreversibly bound to the stationary phase. In this case, both the carrier protein and glycoconjugate peaks showed similar retention behavior, which was not the case for the Thermo HIC-10 column.

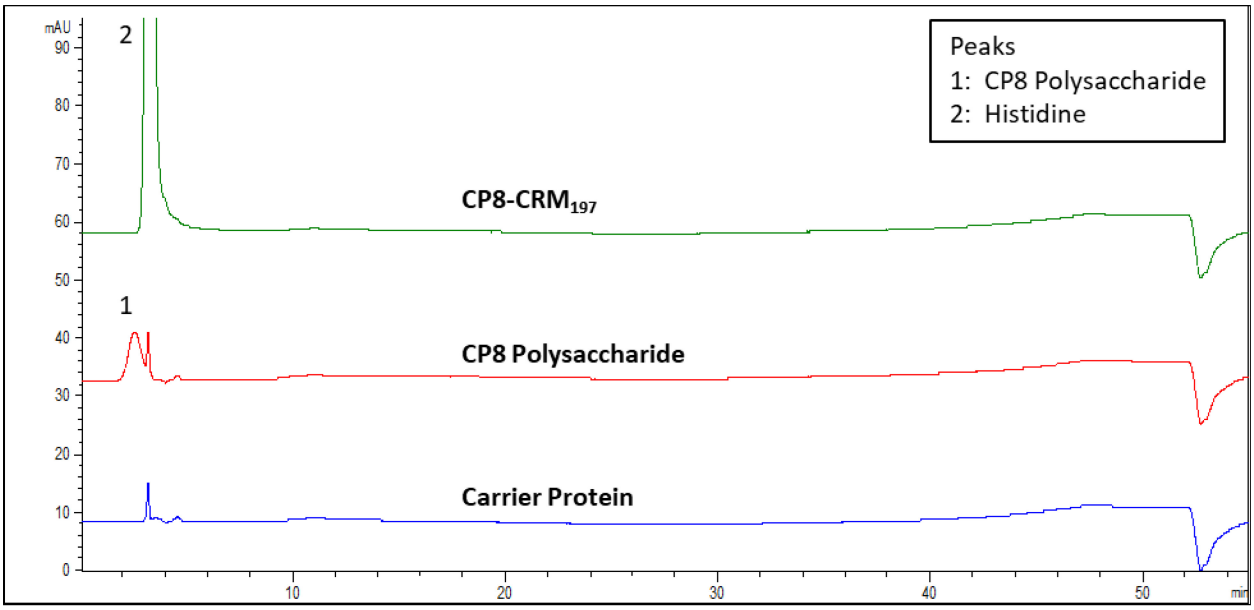

Figure 15 HIC analysis of the carrier protein, CP8 polysaccharide, and CP8-CRM$_{197}$ glycoconjugate using a Tosoh TSKgel Phenyl-5PW column with a descending gradient of 0.75 M ammonium sulfate and ascending gradient of isopropanol in 50 mM potassium phosphate, pH 7.0. The CP8 polysaccharide show slight co-elution with void volume, but with potentially acceptable peak profile. Both the carrier protein and CP8-CRM$_{197}$ failed to elute.

The HIC method conditions with high ionic strength salt and chaotropic modifier on the Tosoh TSKgel Phenyl-5PW column showed promise with improved and potentially acceptable peak shape compared to previous experiments. Even though there is partial co-elution with the void volume, further method development could remedy this issue. However, the hydrophobic character of the carrier protein and glycoconjugate induces tight binding and inability to effectively elute these components. The lack of carrier protein peak prevents the ability to quantitate, and glycoconjugate build up on the column is potentially limiting from a method robustness perspective. These reasons make the method conditions not suitable for the quantitation of free carrier protein and polysaccharide.

2.3.7. SUMMARY

The separation of free polysaccharide and protein proved to be difficult in the 1D format. Most separation parameters related to HIC, such as stationary phase, temperature (data not shown), salt

type, salt concentration, chaotropic modifiers, etc., were explored with little success. As resolution of one component improved, the resolution for another typically suffers, resulting in co-eluting peaks or a poor chromatographic profile. However, through exploration of various modes of separation, several successes and observations were made for the separation of free polysaccharide and protein individually. For example, SEC separates the free carrier protein from the glycoconjugate and free polysaccharide. In addition, RPLC has been demonstrated to separate free polysaccharide from the glycoconjugate and free carrier protein. Denaturing conditions in RPLC were initially avoided due to potential issues with the hydrophobic carrier protein and glycoconjugate. As the RPLC analysis will be intended solely for the quantitation of free polysaccharide, the potential issues with the protein-related components should not impact the analysis. Considering the compatibility of SEC and RPLC mobile phases with SEC mobile phase being a weak solvent in a RPLC separation, coupling these orthogonal modes of separation in a 2D-LC format holds great potential for achieving the desired result.

3. CHAPTER 3: 2D-LC APPROACH TO SEPARATION OF FREE CARRIER PROTEIN AND POLYSACCHARIDE

In this chapter, the development of a 2D-LC approach to separate and quantitate the free carrier protein and polysaccharide in a glycoconjugate vaccine will be presented. 2D-LC has been applied to macromolecules such as recombinant proteins, monoclonal antibodies, and antibody-drug conjugates for a wide array of analyses [19]. However, little to no information has been described in literature for the application of 2D-LC to glycoconjugate vaccines making this a novel application. This work will focus on the development of SEC and RPLC methods, 2D-LC instrumentation design, 2D-LC method development, and assay performance. Performance will then be compared to results from current technologies in place based on capillary electrophoresis (CE) for the quantitation of free polysaccharide and free carrier protein to assess the feasibility of supporting manufacturing process and formulation development studies. The 2D-LC method will be developed for two glycoconjugate drug substances, *S. aureus* serotypes, CP5 and CP8, conjugated to CRM_{197}. In addition, this methodology was explored as a potential platform technology for monitoring free polysaccharide and free carrier protein in other glycoconjugate vaccine projects in the development pipeline.

3.1. AN ALTERNATIVE TO 1D CHROMATOGRAPHY

When 1D chromatography is inadequate in separating peaks of interest, 2D chromatography is a powerful and viable alternative. 2D-LC chromatography involves subjecting a sample to two separation mechanisms. Ideally, the two modes of separation are totally orthogonal in mechanism, which maximizes peak capacity, or the maximum number of components that can be separated in a given separation space [20]. This allows increased selectivity to provide further resolution of components in complex mixtures, such as glycoconjugate vaccines. Several modes

of 2D chromatography commonly used are heart-cutting and comprehensive 2D-LC. The principles of each are illustrated in Figure 16. In heart-cutting 2D-LC, a fraction of one peak (or multiple) is sampled and transferred to the second dimension for further separation. This is a simple and powerful approach for targeted analysis. Also, this decouples the time scale, allowing for longer second dimension gradients if needed. However, the number of components able to be sampled is limited unless additional and more complicated hardware is used. In comprehensive 2D-LC, the entire first dimension eluent is sampled and transferred to the second dimensions for analysis. This mode lends itself to increased complication and instrument requirements as the time scale of the two dimensions is coupled. Therefore, the second dimension separation must be fast enough to keep up with the flow rate of the first dimension while providing adequate efficiency. The second dimension gradient must be completed before the first dimension eluent fills the sampling device (transfer loop). Otherwise, part of the first dimension eluent will overfill the loop and flow to waste resulting in loss of sample and a gap in analysis of the first dimension. Comprehensive mode allows for the most efficient analysis of the components in the first dimension and maximum peak capacity but is the most complicated.

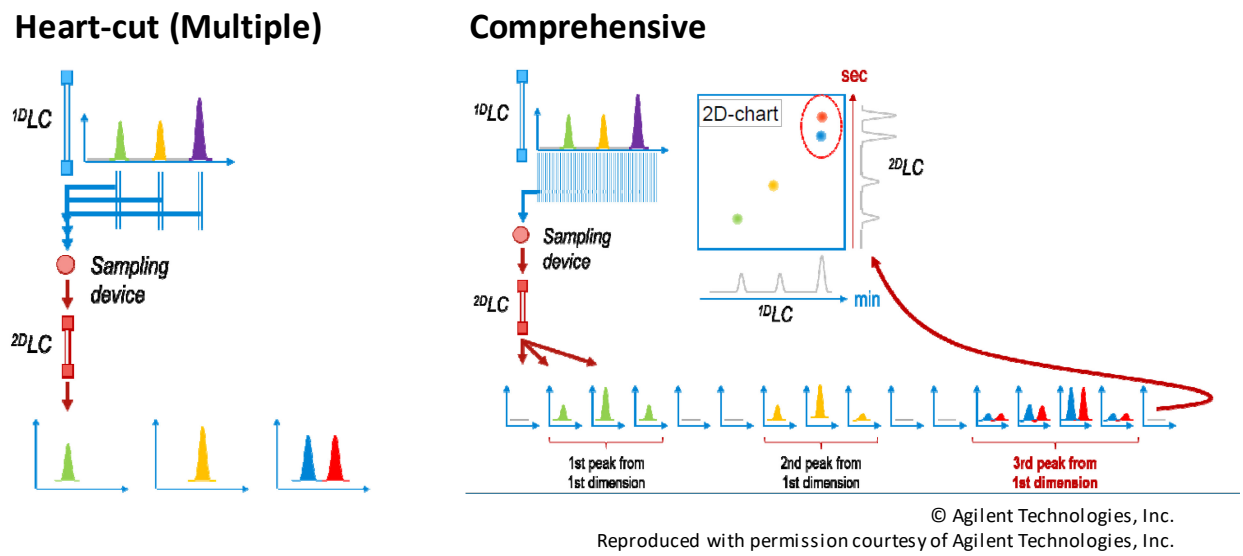

Figure 16. Principles of heart-cut and comprehensive 2D-LC are compared. Both show peaks being sampled from the first dimension and transferred to the second dimension for further separation. Comprehensive samples the entire first dimension while heart-cutting transfers one or more peaks of interest.

The sample mixture, separation needs, and application of the method should be considered when choosing a 2D chromatography mode as there is a trade-off between simplicity and separation power. There are three major components of interest in this glycoconjugate vaccine that need to be separated and quantitated. A single heart-cut 2D-LC separation seemed to be suitable and the most appropriate this application. Therefore, the individual 1D chromatography methods were developed with this end goal in mind. The development focused on optimizing the first dimension for free carrier protein using SEC and the second dimension for free polysaccharide using RPLC.

3.2. INSTRUMENTS

Chromatographic experiments in this work were performed on Agilent 1100 and 1200 HPLC systems (Santa Clara, CA) equipped with a binary/quaternary pump, autosampler, column compartment, and diode array detector (DAD)/variable wavelength detector (VWD). An

Empower 3 data acquisition system was used for controlling the instruments, data acquisition, and analyzing data.

3.3. SAMPLES

Purified reference standards of the individual components were used during the method development in additional to glycoconjugate samples. As relatively low levels of free carrier protein and polysaccharide are present in these final drug substance samples, those components were spiked into the final drug substance to create a spiked glycoconjugate sample. This allowed the free components to be more easily identified and tracked during development. Final spiked levels were approximately 20% for both free carrier protein and polysaccharide. Individual components were diluted to similar working concentrations as the glycoconjugate spike sample and injected individually to confirm and track the free components in the spike sample. Concentrations of protein and polysaccharide were obtained by colorimetric techniques. The protein concentration was determined using the modified Lowry method, and the polysaccharide concentration was determined by the PAHBAH (p-hydroxybenzoic acid hydrazide) assay [15, 16]. As target spike concentrations were calculated from the results of these colorimetric assays, some variability is expected which can explain slight differences in theoretical and experimental results. Samples were transferred to HPLC vials and directly injected for analysis. The samples used are listed in Table 3.

Table 3 Sample Information for 1D Chromatography Development

Name	Concentration (mg/mL)	Preparation
Placebo (L-Histidine)	10 mM	n/a
CP5 Polysaccharide Reference Standard	1.0	Diluted to 0.1 mg/mL in water.
Carrier Protein Reference Standard	5.4	Diluted to 0.1 mg/mL in water.
CP5-CRM_{197} Glycoconjugate	2.0 (1:1 ratio of saccharide: protein)	No preparation.
CP5-CRM_{197} Glycoconjugate Spike	0.5 (1:1 ratio of saccharide: protein)	Glycoconjugate sample spiked with saccharide and carrier protein at ~20% free saccharide and carrier protein.

The method development focused on one serotype for the majority experiments. The purpose was to increase efficiency as the structures of the two serotypes are similar. Therefore, the physical/chemical characteristics exploited for separation were not expected to differ greatly between the serotypes.

3.4. Development of First Dimension Method for Free Carrier Protein by SEC

The focus of developing the first dimension SEC method was to achieve enough resolution of the carrier protein from the other glycoconjugate components to allow quantitation of the carrier protein. The separation of the glycoconjugate and polysaccharide will be performed in the second dimension separation and therefore discussed in the next section. As previously stated in section 1.2, SEC alone has been shown to be inadequate in resolving both free protein and polysaccharide from the glycoconjugate. This was confirmed during development experiments while attempting to reproduce observations in literature as well as help understand the separation mechanism observed with the Thermo HIC-10 column. The glycoconjugate, polysaccharide, and carrier protein were individually injected onto a Tosoh G2000 SWxl SEC column (7.8x300 mm, 5 µm, 125 Å porosity, part # 08540) and isocratically separated with 50 mM sodium sulphate, 20 mM sodium phosphate, pH 6.8 at a flow rate of 0.75 mL/min. Peaks were detected using a UV diode array detector monitoring both 210 nm for all components and 280 nm for protein-related peaks. An overlay of the three injections are shown in Figure 17. Carrier protein is adequately

resolved from the glycoconjugate and polysaccharide. As expected, the polysaccharide and glycoconjugate co-elute due to similar effective hydrodynami radii. The separation is adequate for monitoring free protein in glyconjugate samples, but further development would be needed to ensure SEC was suitable for quantitation of the carrier protein.

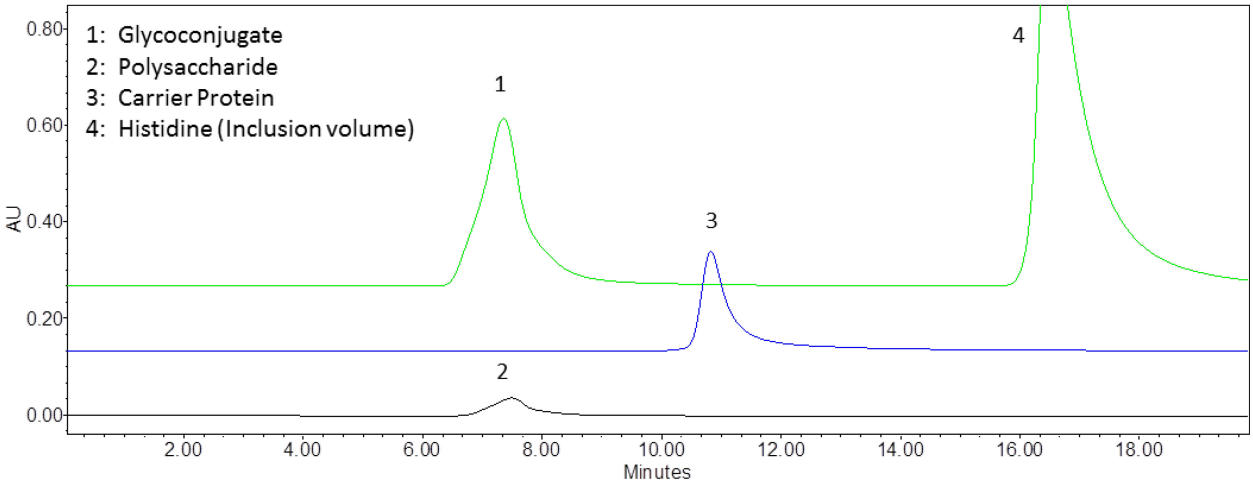

Figure 17. SEC-HPLC analysis of a glycoconjugate sample (top), carrier protein (middle), and polysaccharide (bottom) using a Tosoh G3000 SWxl column (4.6x150 mm, 5 µm, 125 Å) showing co-elution of glycoconjugate and polysaccharide peaks. The mobile phase was 50 mM sodium phosphate, pH 6.3 and peaks were detected at UV 214 nm.

SEC was unsuccessful in resolving all three components, glycoconjugate, polysaccharide, and carrier protein, however, the experiment performed was critical in finding separation conditions capable of adequately resolving free carrier protein from the others. These results served as a basis to evaluate RP-HPLC as the second dimension, with SEC serving as the first dimension. Modifications to the SEC separation conditions (used in Figure 17) were necessary for optimization and compatibility in a 2D-LC format. In 2D-LC, smaller peak volumes are desired as peaks are sampled from the first dimension and transferred to the second dimension. This minimizes peak dispersion and column equilibrium disruptions in the second dimension that can increase total analysis time and potentially result in under recovery of the target analyte. Also, smaller peak volumes allow the usage of commercially available loops with fittings that are more

compatible for 2D-LC-specific switching valves operating under high pressure conditions. The SEC column was evaluated first as column dimensions are a critical factor in determining sampling time and peak transfer volume. A Waters Acquity UPLC column (Part #186005225) was evaluated to utilize the smaller ID column and higher pressure limit to reduce analysis time and peak volumes. The glycoconjugate, polysaccharide, and carrier protein were directly injected for analysis. Column information and method parameters are listed in Table 4 and an overlay of the individual components is shown in Figure 18.

Table 4. SEC Method Conditions using Waters UPLC Column

Parameter	Setting
Column	Waters UPLC Acquity Protein BEH, 4.6 x 150 mm, 1.7 µm, 200 Å, Part #186005225
Mobile Phase A (MPA)	30 mM Sodium Phosphate[1], 150 mM Sodium Chloride, 0.1 mM EDTA, pH 6.7
Flow Rate	0.5 mL/min
Injection Volume	5 µL
Column Temperature	30° C
Elution Gradient Program	Isocratic
UV Wavelength	210 and 280 nm

[1] Dibasic sodium phosphate heptahydrate and monobasic sodium phosphate monohydrate used

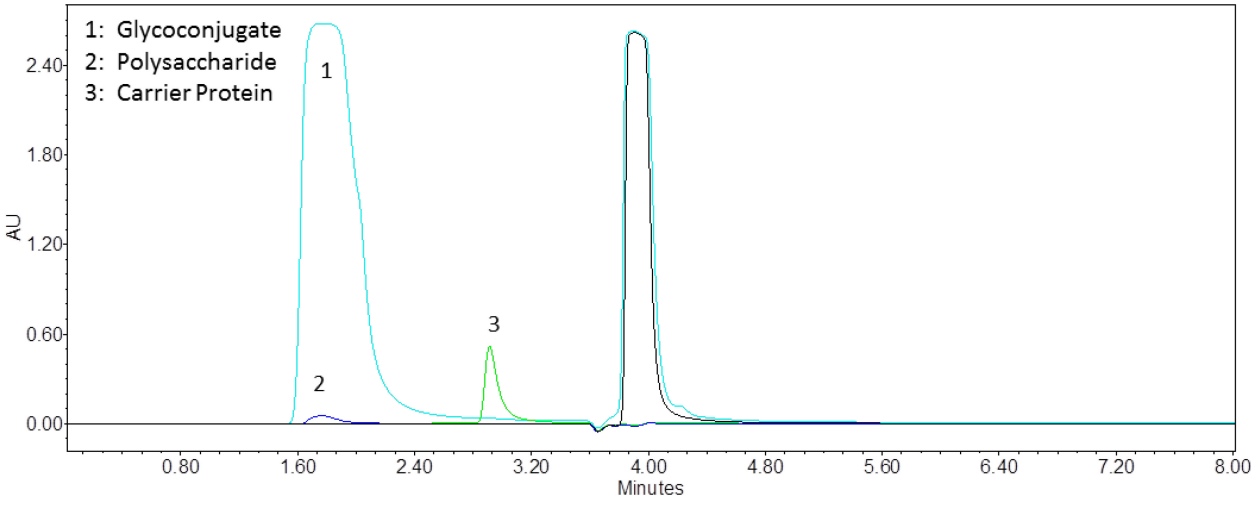

Figure 18. SEC-HPLC analysis of individual glycoconjugate components using a Waters Acquity UPLC BEH column shows comparable separation to previous SEC separation with Tosoh G3000 SWxl.

The overlay of the individual components shows the Waters UPLC column was able to achieve a similar separation profile in approximately 40% the time compared to the Tosoh SEC column in

a conventional HPLC format (Figure 17). Also, using the peak width (W_b) and flow rate (mL/min) to calculate peak volume, the volume of the saccharide peak was reduced from approximately 2.8 mL (Tosoh) to 0.3 mL (Waters UPLC). The reduction in peak volume was desirable as this significantly minimized the volume of polysaccharide and glycoconjugate to be transferred to the second dimension. Therefore, the Waters UPLC column was used going forward in all future SEC development experiments.

3.4.1. COLUMN LOAD

An initial screening of column load linearity of the carrier protein was performed and the impact of increasing injection volume and glycoconjugate concentration was assessed. The glycoconjugate spike of sample with approximately 20% free carrier protein was injected at 1, 3, and 5 µL targeting 0.1, 0.3, and 0.5 µg column loads for free carrier protein. Increasing the injection volume also increased the column load of the glycoconjugate at 0.5, 1.5, and 2.5 µg protein. Injection volumes of 1 – 5 µL were chosen as column manufacturer recommendations are <20 µL. Knowing the large size of the glycoconjugate, caution was taken to minimize column loads and prevent damage to the column. An overlay of the three injections is shown in Figure 19. Resolution between the glycoconjugate and carrier protein peaks appeared adequate and consistent for the range of column loads tested.

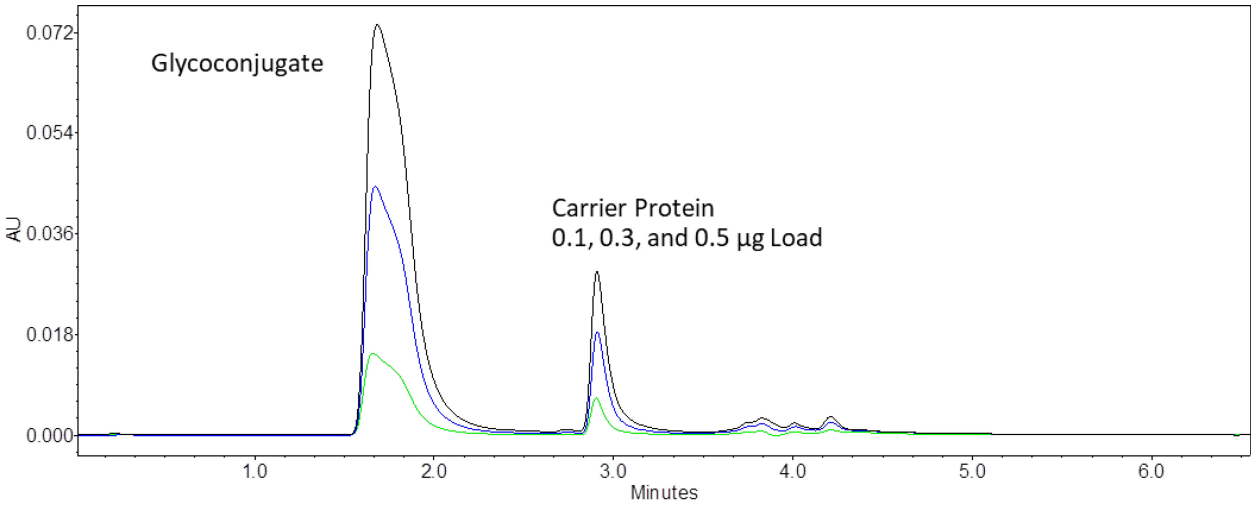

Figure 19. SEC-HPLC analysis of glycoconjugate spike sample using a Waters Acquity UPLC BEH column with varying column loads, 0.1 µg (green), 0.3 µg (blue), and 0.5 µg (black). Mobile phase was 30 mM sodium phosphate, 150 mM sodium chloride, 0.1 mM EDTA and peaks were detected at UV 280 nm

The carrier protein peak was then integrated, and peak area was used to calculate response factors across the range using the theoretical column load. The data is shown in Table 5, and response factors appear similar across the column load range tested. In addition, the response factor is plotted along with a linear regression of peak area vs. column load. The linear plot shows a good linear fit with an R-squared of 1.00, and the response factor shows no obvious bias across the range tested.

Table 5. Area count and response factor plot data of carrier protein peak from glycoconjugate spike sample injections in Figure 19.

Injection Volume (µL)	Glyconconjugate Column Load (µg protein)	Carrier Protein Column Load (µg)	Area (µV*sec)	Response Factor (Area/Load)
1	0.5	0.1	41825	418250
3	1.5	0.3	124834	416113
5	2.5	0.5	209011	418022

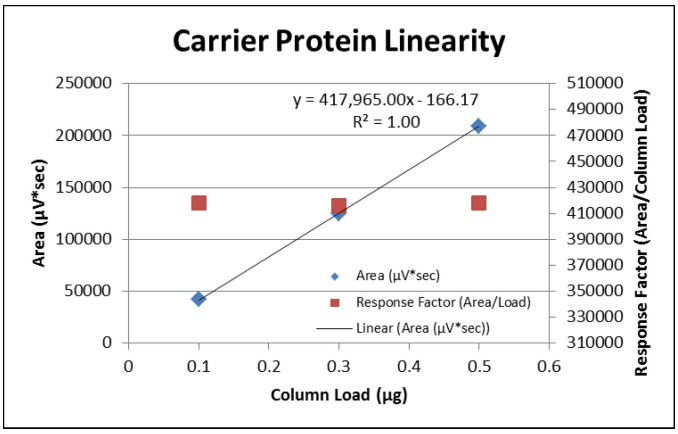

Figure 20. Area and response factor data from Table 1 plotted against column load showing a good linear fit.

For initial screening purposes, limited points were tested with ranges on the lower end of the expected column load for test samples, but the data provided useful information in that the glycoconjugate peak exhibited no obvious signs of negatively impacting recovery of the carrier protein. A broader range of carrier protein column load with higher glycoconjugate concentrations was evaluated during the qualification of the final method.

3.4.2. FLOW RATE

The effect of flow rate on the SEC separation of the glycoconjugate components was assessed. Decreasing the flow rate could potentially gain more resolution between the glycoconjugate and carrier protein and allow more flexibility in timing of the valve switch for peak transfer to the second dimension. The glycoconjugate spike sample was injected at 3 µL onto the Waters BEH column using a flow rate of 0.5 and 0.3 mL/min. An overlay of the two injections is shown in Figure 21. By SEC analysis, CRM_{197} can have measurable amounts of high molecular mass species (HMMS). The HMMS was observed and integrated at 2.7 minutes in Figure 21A and 4.4 minutes in Figure 21B. Therefore, resolution (R) was calculated using the HMMS peak as it elutes closer to the glycoconjugate peak. Resolution for both flow rates were 1.7 and 1.6, for 0.5 and 0.3 mL/min, respectively, indicating the flow rates tested showed little impact on the

resolution. With a resolution of greater than 1.5 and the small percentage of HMMS present in the sample, this was acceptable using either flow rate tested.

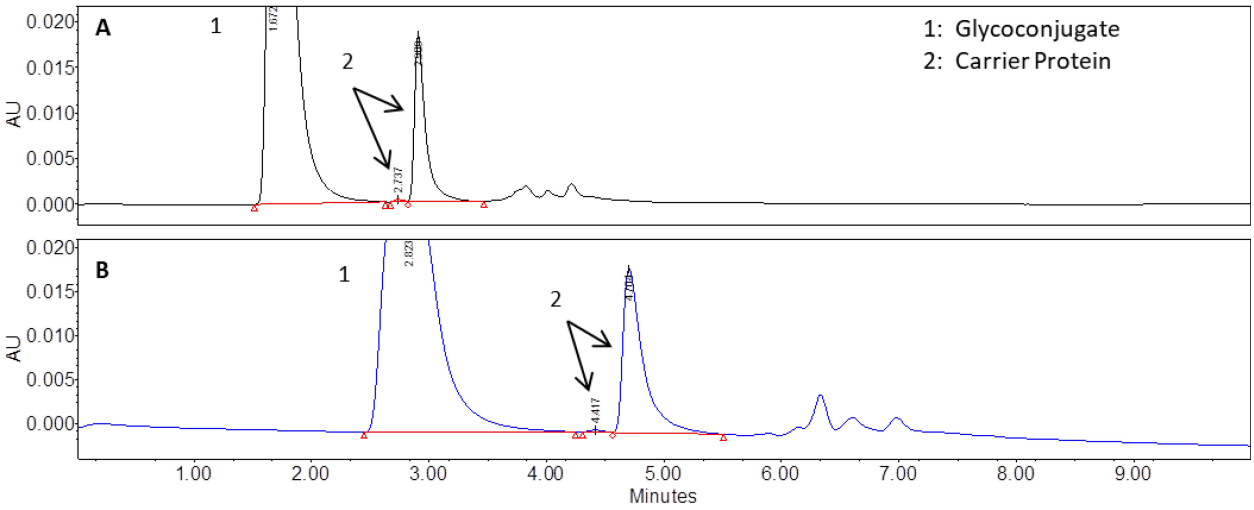

Figure 21. SEC-HPLC analysis of glycoconjugate spike sample using a Waters Acquity UPLC BEH UPLC column with varying flow rates: A) 0.3 mL/min. and B) 0.5 mL/min. Mobile phase was 30 mM sodium phosphate, 150 mM sodium chloride, 0.1 mM EDTA pH 6.7 and peaks were detected at UV 280 nm.

3.4.3. MOBILE PHASE SCREENING

Properties of the stationary phase in an SEC column can be different depending on the base support used, such as polymer or silica based, and can vary vendor-to-vendor due to specific manufacturing processes. Therefore, various mobile phases were screened to optimize separation between the glycoconjugate and carrier protein and potentially improve peak shape by suppressing any secondary interactions with the stationary phase. Mobile phases were prepared with different salts, increasing sodium chloride concentration, and the addition of organic modifiers. The mobile phases used are listed in Table 52 (A – E). The separation of glycoconjugate and carrier protein using each mobile phase is shown in Figure 22.

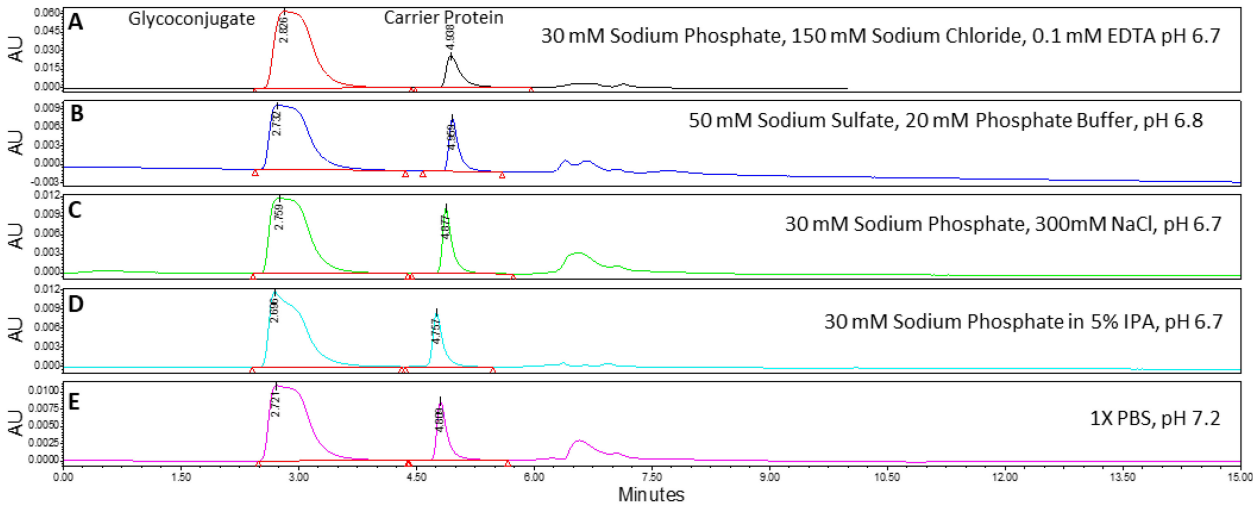

Figure 22. Mobile phases screened for SEC-HPLC analysis of glycoconjugate spike sample using a Waters Acquity UPLC BEH UPLC column; A) initial B) sulfate salt C) increased NaCl D) organic modifier E) commercial PBS. Peaks were detected at UV 280 nm.

Note, in Figure 22-A, the sample was injected targeting a 0.5 µg column load compared to a 0.1 µg column load in the other chromatograms. The column load is within the previously tested range and should be adequate for optimizing the relative retention time between these two peaks and evaluating area counts. All mobile phases evaluated appeared to give similar relative retention times between the two peaks of interest. Area counts for the carrier protein were also similar across mobile phases evaluated. Any choice from the mobile phases tested will give adequate separation, but the mobile phase with the lowest ionic strength and salt concentration will be desired to minimize the disturbance of the baseline in the RPLC second dimension for the separation of free polysaccharide.

3.4.4. Particle Pore Size

The last SEC attribute optimized was the porosity of the column to potentially gain additional separation of the carrier protein (molecular weight of 58k Daltons) peak from the polysaccharide and glycoconjugate peaks. Separations previously discussed with the Waters BEH column used a pore size of 200 Å. Pore sizes of 125 and 450 Å were evaluated using the same Waters BEH

chemistry to maintain consistency with previous experiments. The column specifications are listed in Table 6.

Table 6. Waters Acuity UPLC Protein BEH SEC Column Specifications

Part #	Particle Size (µm)	Pore size (Å)	Dimensions (mm)
186006505	1.7	125	4.6 x 150
186005225	1.7	200	4.6 x 150
186006851	2.5	450	4.6 x 150

The polysaccharide and carrier protein were individually injected at 1 µL onto both the 125 and 450 Å Waters UPLC BEH columns and isocratically separated with mobile phase B in Table 52 at 0.5 mL/min. The peaks were detected using UV detection at 214 nm in order to monitor both components as the polysaccharide has low absorbance at 280 nm. An overlay of the two components is shown in Figure 23 using the 125 Å column. The smaller pore size showed a decrease in retention time of the carrier protein peak, shifting closer to the polysaccharide peak. The two components are resolved and the carrier protein within the calibration range of column (1 – 80 kDa); however, the loss of resolution between the components could lead to robustness issues over time impacting carrier protein recovery.

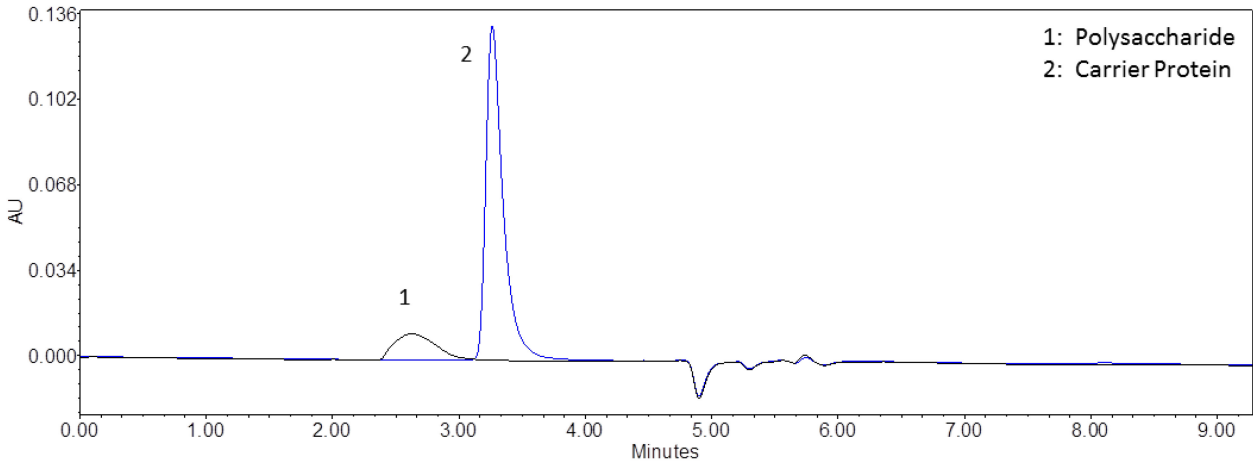

Figure 23. SEC-HPLC analysis of polysaccharide and carrier protein individually injected onto a Waters Acquity UPLC BEH column with a pore size of 125 Å. Mobile phase was 30 mM sodium phosphate, 150 mM sodium chloride, 0.1 mM EDTA and peaks were detected at UV 214 nm.

An overlay of the two components is shown in Figure 24 using the 450 Å column. The larger pore size showed an increase in carrier protein peak retention time large enough to partially co-elute with the inclusion volume. This could have been predicted as the molecular weight of the carrier protein falls below the calibration range of the column (100 – 1500 kDa). Also, an interesting observation was the significant increase in tailing observed with the polysaccharide peak. There could be several causes for this tailing, such as secondary interactions of the poly saccharide with the stationary phase, or given the heterogeneity in the molecular weight distribution of the polysaccharide, the smaller molecular weight species having greater access to the 450 Å pore and further separating.

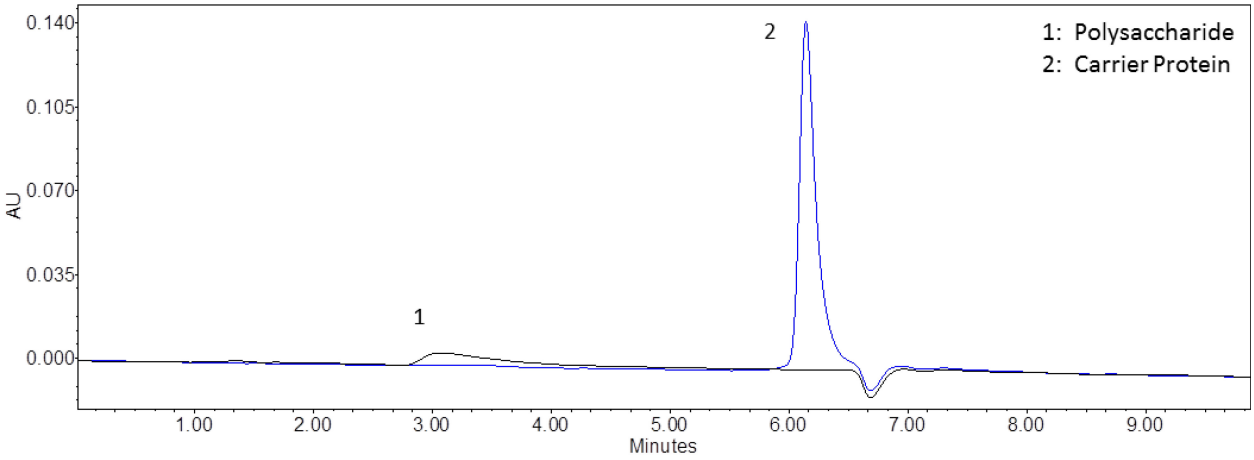

Figure 24. SEC-HPLC analysis of polysaccharide and carrier protein individually injected onto Waters Acquity UPLC BEH column with a pore size of 450 Å. Mobile phase was 30 mM sodium phosphate, 150 mM sodium chloride, 0.1 mM EDTA and peaks were detected at 214 nm

For the purpose of optimizing pore size for the carrier protein, the polysaccharide sufficed as a comparator as its hydrodynamic radius has been shown to be similar to that of the glycoconjugate. The results provided in sections 3.4.1 to 3.4.3 show the 200 Å pore size appears to be the ideal choice within the Waters UPLC BEH columns based on the relative retention of the components observed. In addition, the results are consistent with what literature predicted

based on the calibration curves of the columns (provided by Waters). Therefore, the 200 Å pore size will be taken forward for the first dimension SEC separation.

In summary, the finalized SEC method conditions to be applied to the first dimension 2D-LC separation are listed in Table 7.

Table 7. Developed SEC Method Conditions using Waters UPLC Column

Parameter	Setting
Column	Waters UPLC Acquity Protein BEH, 4.6 x 150 mm, 1.7 µm, 200 Å, Part #186005225
Mobile Phase A (MPA)	30 mM Sodium Phosphate, 150 mM Sodium Chloride, 0.1 mM EDTA, pH 6.7
Flow Rate	0.3 mL/min
Injection Volume	5 µL (sample)
Column Temperature	30° C
Elution Gradient Program	Isocratic
UV Wavelength	280 nm
Run Time	10 min.

3.5. Development of Second Dimension Method Free Polysaccharide by RPLC

The first dimension SEC conditions were evaluated and selected in the previous section. Next, a separation of the polysaccharide and glycoconjugate peaks would be needed that is also compatible with an aqueous first dimension. RPLC was a natural choice for the second dimension mode of separation for several reasons that include low ionic strength SEC mobile phases are miscible in RPLC buffers and an aqueous SEC mobile phase is a weak solvent in a RPLC separation. In addition, similar work performed by He *et al* has shown feasibility with glycoconjugate sample types. This section will describe the development of the second dimension method, highlight unique challenges with glycoconjugate samples, and provide the solutions implemented to overcome these challenges.

3.5.1. Column Selection and Screening of Glycoconjugate Peaks

Most importantly, an RPLC method capable of binding and eluting the polysaccharide would be needed as the second dimension separation. The ability to retain the polysaccharide overcomes two major issues in 2D chromatography, baseline disturbances and peak broadening. Retaining the polysaccharide allows time for the second dimension to re-establish equilibration as well as eliminates peak broadening from the first dimension by focusing the analyte at the head of the column. An Agilent Zorbax StableBond 300 CN (4.6 x 150 mm, 3.5 µm, part# 863973-905) RPLC column was first chosen. The cyano (CN) stationary phase seemed well suited for this application with its low hydrophobicity and unique dipole charge characteristics, which ideally would minimize interaction with the hydrophobic glycoconjugate and increase retention of the polysaccharide. Method conditions for this experiment are shown in Table 8. A screening gradient was programmed from 1 – 80% mobile phase B (MPB) to evaluate elution conditions for the polysaccharide and glycoconjugate. Isopropanol being a strong organic solvent in RPLC was chosen to help elute the hydrophobic glycoconjugate. Trifluoroacetic acid (TFA) was added to both mobile phases as an ion-pairing agent to help minimize non-specific interactions with the stationary phase and improve peak shape. Polysaccharide and the glycoconjugate spike sample were individually injected at 7 µL to target a polysaccharide column load of approximately 1 µg. Lower column loads were intentionally targeted to minimize damage to the column from the glycoconjugate clogging the inlet frit or irreversible binding to the stationary phase, and to mimic lower concentrations to be transferred to the second dimension.

Table 8. RPLC Method Conditions for Initial Screening of Free Polysaccharide

Parameter	Setting
Column	Agilent Zorbax StableBond 300 CN (4.6 x 150 mm, 3.5 µm)
Mobile Phase A (MPA)	0.20% TFA in Water
Mobile Phase B (MPB)	0.15% TFA in Isopropanol

Flow Rate	0.5 mL/min
Column Temperature	30° C
Elution Gradient Program	1 – 80% MPB in 20 min, hold 80% MPB for 5 min., return to 1% MPB and hold for 10 min.
UV Wavelength	214 nm

An overlay of the chromatograms in Figure 25 shows adequate retention and resolution of the polysaccharide (peak 1) and glycoconjugate (peak 2). Interestingly, the free carrier protein (peak 3) is nearly resolved from the glycoconjugate. However, resolution decreased at higher glycoconjugate column loads, which deterred pursuing further development. Therefore, free polysaccharide analysis remained the sole focus of the RPLC experiments as separation of free carrier protein has already been achieved by SEC.

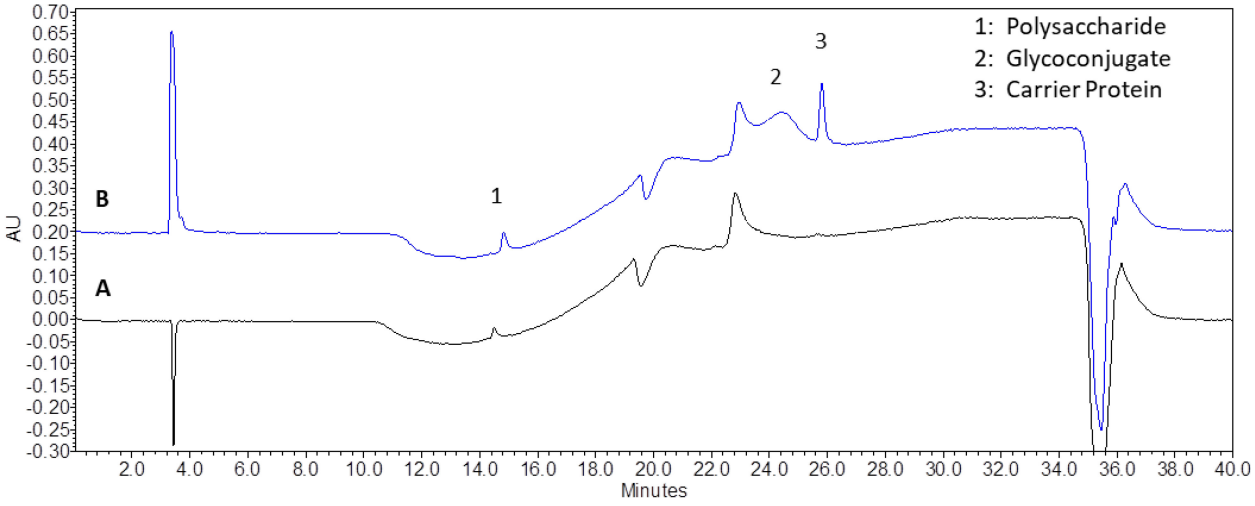

Figure 25. Initial RPLC gradient screening with samples A) polysaccharide B) glycoconjugate spike, individually injected onto an Agilent Zorbax SB300 Cyano column. Peaks were eluted with an ascending gradient of isopropanol with TFA, and peaks were detected at UV 214 nm.

3.5.2. Optimization of Gradient for Elution of Polysaccharide

Adequate resolution of the polysaccharide from the other glycoconjugate components had been achieved. Next, experiments were performed to modify the gradient to minimize baseline slope and increase efficiency of the separation. Using the gradient and retention time, the polysaccharide eluted at approximately 26% MPB under initial conditions. Therefore, the same method conditions from Table 8 were used except for 10% MPB in the initial gradient

conditions, which effectively reduced the gradient slope. The glycoconjugate spike sample was injected at 7 µL (0.7 µg polysaccharide column load) and analyzed under the initial and modified gradient conditions. The glycoconjugate spike sample analyzed by RPLC under both MPB starting conditions are shown in Figure 26. This turned out to be a critical experiment as a response from the polysaccharide was observed prior to the void volume indicating retention was not strong enough under the 10% MPB starting conditions. In a separate experiment, the polysaccharide was also observed eluting prior to the void volume under isocratic conditions using 20% MPB (data not shown). The combination of the stationary phase and polysaccharide both having relatively low hydrophobicity resulted in weak retention of some polysaccharide species. Rather than opting for a more hydrophobic stationary phase, a lower percentage of MPB was maintained as a more hydrophobic stationary phase could result in strong and potentially irreversible binding of the glycoconjugate. For the purpose of quantitation, peak recovery is paramount, and gradient optimization can be achieved by optimizing other parameters.

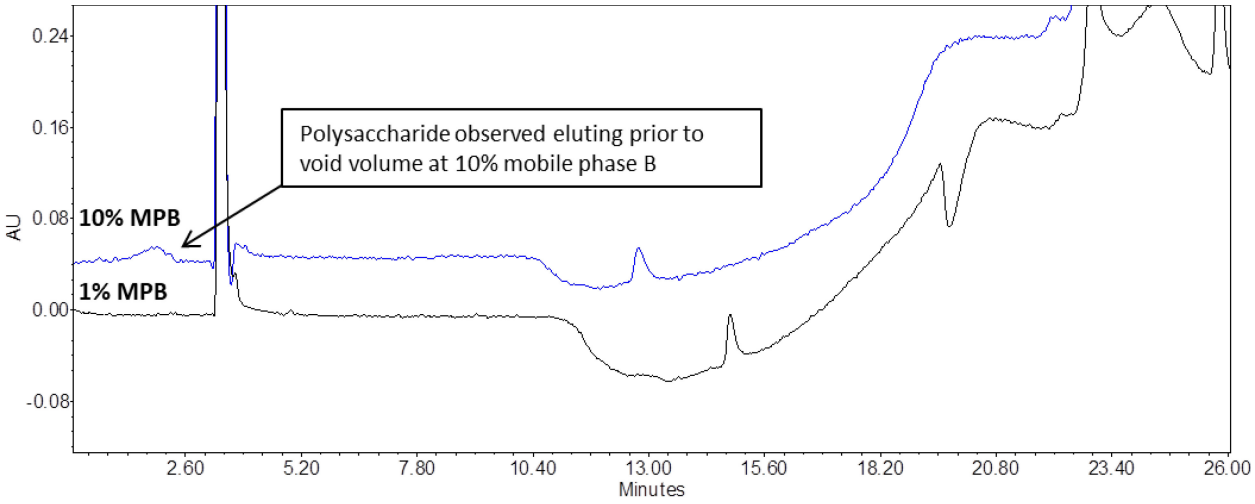

Figure 26. RPLC analysis of the glycoconjugate spike sample with an Agilent SB300-cyano column using gradients starting with 1 and 10% mobile phase B. Peaks were eluted with an ascending gradient of isopropanol and TFA were detected at UV 214 nm. Early elution of the polysaccharide observed before the void volume using the gradient starting with 10% mobile phase B.

Since the polysaccharide showed sensitivity in retention to increases in the percent organic solvent, other parameters were explored to improve the chromatographic profile and efficiency of the separation. The % TFA in mobile phase A and B were reduced and optimized to better match refractive properties of the ion pairing agent. As the polysaccharide is the main component of interest, the ending %MPB was decreased and gradient time reduced. The updated method conditions are listed in Table 9.

Table 9. Updated RPLC Method Conditions for Free Polysaccharide

Parameter	**Setting**
Column	Agilent Zorbax SB 300 CN (4.6 x 150 mm, 3.5 µm)
Mobile Phase A (MPA)	0.10% TFA in Water
Mobile Phase B (MPB)	0.085% TFA in Isopropanol
Flow Rate	1.0 mL/min
Column Temperature	30° C
Elution Gradient Program	Hold at 5% MPB – 5 min., then 5 – 40% MPB for 5 min., increase and hold at 80% MPB for 2.5 min., return to 5% MPB and hold for 8 min.
UV Wavelength	214 nm

The analysis of a glycoconjugate spike sample is shown in Figure 27. The updated conditions produced less baseline slope with improved peak shape. Also, the run-time was reduced by half, which significantly helps minimize overall analysis time in a 2D-LC format.

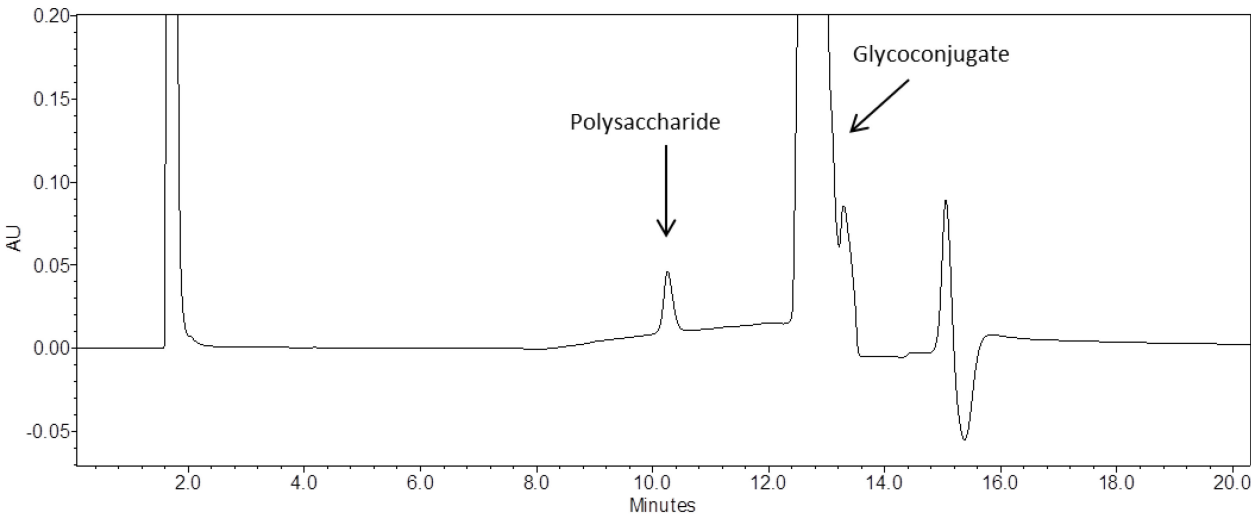

Figure 27. RPLC analysis of the glycoconjugate spike sample with an Agilent SB300-cyano column using optimized conditions.

3.5.3. LINEARITY EVALUATION OF POLYSACCHARIDE

Before coupling the RP-LC method in a 2D-LC format, linearity was performed as a pre-qualification experiment. This was to help ensure method performance without impact from the first dimension SEC or switch-valve instrumentation as difficulties can arise when investigating method issues in a 2D-LC format. Linearity was evaluated by analyzing the polysaccharide standard at column loads ranging from 0.1 – 3.0 µg. Using a 0.1 mg/mL polysaccharide stock, the injection volume was adjusted to achieve target column loads. A response factor plot in Figure 28 was generated using the polysaccharide area count and column load with limit bars at 90 and 110% of the average response factor across the range for reference. A ±10% response factor limit seemed to be an appropriate guideline in assessing linearity for a quantitative method.

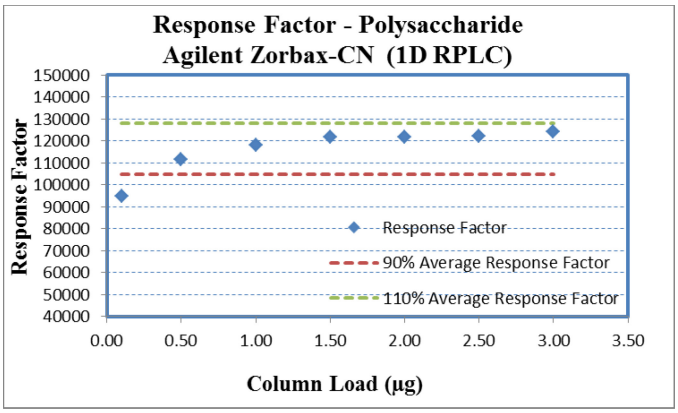

Figure 28. Response factor of the polysaccharide plotted against column load using the Zorbax-CN. An upward trend in response factor was observed as column load increased.

An upward trend in response factor was observed as column load increased, especially at the lower concentrations, indicating the response was increasing across the range tested. This could be due to the polysaccharide interacting with the silica base support resulting in decreased recovery. However, all points above a 0.5 µg column load were within 10% of the average response factor. Therefore, the method conditions listed in Table 9 appear to be suitable for resolving polysaccharide from other glycoconjugate components.

Additional qualification experiments, such as precision, accuracy, and quantitation limit, were planned to be executed in a 2D-LC format. At this point, conditions had been developed for the separation of free carrier protein by SEC (section 3.4) and free polysaccharide by RPLC (section 3.5). The next major step in the project was to couple these separation modes together to develop a functional 2D-LC method.

3.6. Development of 2D-LC Method

3.6.1. 2D-LC Instrumentation Design and Method Development

Designing and developing a 2D-LC method requires choosing an appropriate instrumentation configuration based on the needs of the application. Several options can be taken to achieve a

fully functional 2D system. The most economical option is simply coupling two HPLCs via an existing switching valve. This option is the most cost-effective to implement in an R&D laboratory and has been proved functional and effective, but there are pitfalls to be aware of. This approach requires manual control over critical method events, such as valve switching, gradient start, transfer volume, etc., that must be programmed into separate instrument methods. This can be problematic when method changes upstream in the flow path are not compensated for downstream. Also, integrating 2D-specific software for data analysis, which is a powerful tool when analyzing more complex 2D-LC separations, is not always possible.

An alternative option to achieve a functional 2D-LC system is purchasing a commercially available system. A complete commercial system can be considerably more expensive than a manually built system, but the commercial system provides several significant advantages. Firstly, a commercial system is typically equipped with a 2D-specific switching valve, which is designed to handle high frequency switching demanded by 2D-LC methods therefore increasing robustness. Secondly, 2D-specific software integrated into the system can simultaneously controls both dimensions. This allows for easier method programming as the software precisely controls critical method events for both dimensions resulting in less programming errors and time spent trouble-shooting. The software also allows for more efficient and powerful data analysis with visualization tools for more complex separations. Lastly, a commercial system is a more practical option to implement into a cGMP environment where a method must be transferrable and have consistent control over method parameters. For the advantages listed above, a commercial 2D-LC system seemed ideal for applications in vaccine research and development.

Initial Instrument Configuration and Setup Requirements (2D-LC Feasibility)

Agilent Inc. provided a demo 2D-LC instrument for this project as an evaluation of its application to vaccine research and development. The Agilent instrument was an Infinity II series equipped with a 1290 high speed binary pump, diode array detector (DAD), and an 8-port/2-position switching valve. The demo modules were coupled and integrated to an existing Agilent 1260/1290 HPLC. Agilent's 2D-LC software was utilized to control the instrument, program methods, and analyze data.

The 8-port/2-position switching valve is what links the two dimensions together. This switching valve is equipped with two symmetrical flow-paths and sample loops that can be configured in a parallel or counter-current flow for flushing loops. These attributes can aid in retention consistency and reduce sample loss in the second dimension. A diagram of the switching valve flow is shown in Figure 29. In position 1, eluent from the first dimension column flows to the first loop, while mobile phase from the second dimension pump flows to the second loop and second dimension column. In position 1, analysis is being performed in the first dimension while the second dimension column is equilibrated by the second pump awaiting sample transfer. Once the peak of interest enters the loop, the valve switches to position 2. In position 2, the peak of interest will flow to the second dimension column and a gradient will be performed for further separation and analysis. While this occurs, eluent from the first dimension then begins to fill the second loop.

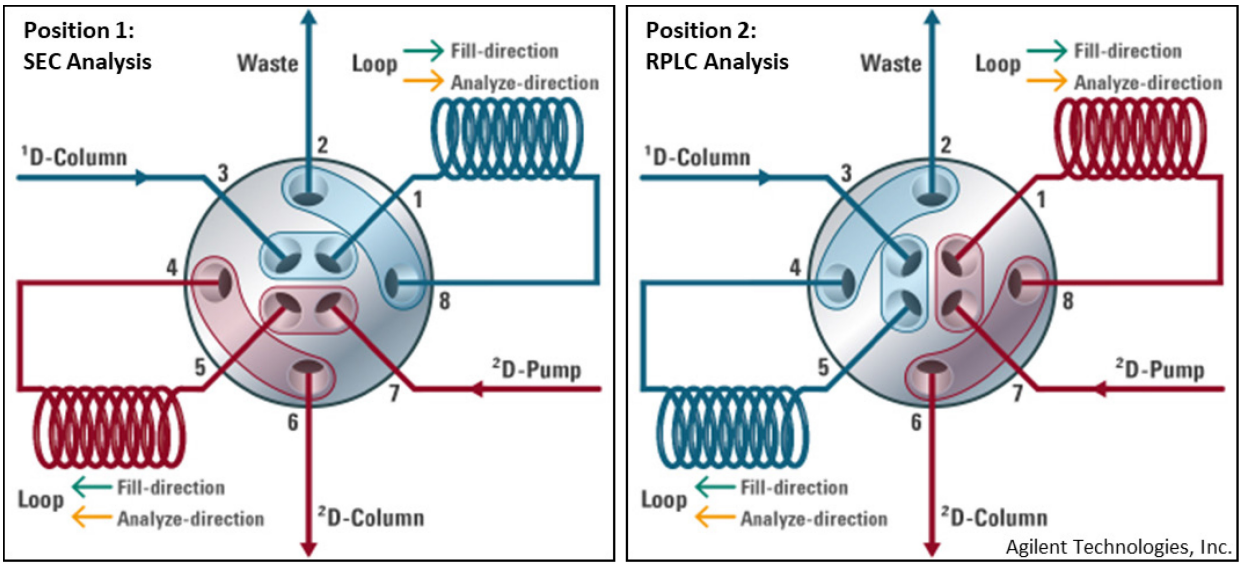

Figure 29. Two positions of 1290 Infinity Valve Drive (8-port/2-position). Position 1 used for SEC analysis. Once peaks enter the loop, the valve switches to position 2 and transfers peaks to second dimension for RPLC analysis.

In a single heart-cut mode, only one loop is utilized with a single valve switch, and the full analysis is finished once the second dimension gradient completes. In comprehensive 2D-LC mode, once the second dimension gradient is completed (typically several sec.) the valve will switch back to position 1, sending eluent from the second loop to the second dimension for further analysis. The switching process repeats continuously over the course of the injection, effectively sampling the entire first dimension eluent. Multiple heart-cut mode also performs multiple valve switches utilizing both sample loops, but there are timed delays to allow the next peak of interest to enter the second sample loop for a more targeted analysis.

Although a single-heart cut was intended for the application of free carrier protein and polysaccharide, the instrument was initially delivered and installed in a configuration intended for comprehensive 2D-LC. Therefore, initial feasibility experiments were performed using the instrument in a comprehensive 2D-LC format to ensure proper instrument start-up. In this configuration, small loops (40 µL) were installed on the switching valve coupled to a high-speed

gradient in the second dimension for increased sampling across peaks in the first dimension. This required modifications to the previously developed SEC and RPLC methods discussed in the sections 3.4 and 3.5. The flow rate in the first dimension SEC separation was reduced from 0.3 to 0.1 mL/min. In addition, to speed up the second dimension RPLC separation, the flow rate was increased from 1.0 to 1.5 mL/min., and the entire gradient was reduced to 0.5 min. This required the use of a shorter, UPLC cyano column (Agilent Poroshell EC-CN) to reduce back-pressure. These changes increased the sampling rate across the first dimension and minimized sample loss due to overfilling the switching-valve loop. Targeting a transfer volume of 25 – 75% of the loop volume is a safe range to prevent sample loss from under or over filling a sample loop. However, even with the method conditions described above, the loop fill was 125%, which resulted in slight loss of sample volume from the first dimension. Though not ideal, this was acceptable for demonstrating feasibility of the 2D-LC separation. The updated method conditions for feasibility are shown in Table 10.

Table 10: 2D-LC Instrumentation and Method Parameters for Feasibility Experiments

Parameter	**1st Dimension – SEC**	**2nd Dimension - RPLC**
Pump	1260 Infinity Quaternary G5611A	1290 Infinity II Binary G7120A
Autosampler	1290 Infinity (G4226A)	
Detector	1260 Multi-wavelength (MWD) G1365D	1290 Infinity II Diode Array G7117B
Valve	1290 Infinity Valve Drive (8-port/2-position) G1170A	
Valve Loop volume	40 µL	
Column	Waters Acquity UPLC BEH, 4.6 x 150 mm, 1.7 µm (186005225)	Agilent Poroshell EC-CN 4.6x50mm, 2.7 µm (699775-905)
Mobile Phase	30 mM Sodium Phosphate, 150 mM Sodium Chloride, 1 mM EDTA, pH 6.7	MPA: 0.10% TFA in Water MPB: 0.085% TFA in Acetonitrile
Flow Rate	0.1 mL/min	1.5 mL/min
Gradient	Isocratic	2 – 70% MPB for 0.29 min., return to 2% at 0.30 min. and hold for 0.20 min.
Detection Mode	UV 280 nm	UV 214 nm
Column Temp.	30° C	40° C
Valve Configuration	See Figure 29	
Valve Switch Time	Every 0.5 min. from 6 – 17 min. (Comprehensive)	
Injection Volume	6 µL	
Total Runtime	25 min	

The goal of these feasibility experiments was to successfully transfer the co-eluting glycoconjugate and polysaccharide peaks to the second dimension, then separate and detect them similarly to the 1D methods developed in the previous sections. For simplicity, the CP5 polysaccharide reference standard was first analyzed using the method conditions listed in Table 10. The 2D-LC analysis of the polysaccharide standard is shown in Figure 30 with the first dimension SEC overlaid with the second dimension RPLC showing the sampling across the polysaccharide peak. The first dimension eluent was sampled from 6 – 17 min. and subjected to further analysis by RPLC with a 0.5 min ballistic gradient (shown within Figure 30). This allowed continuous sampling across the entire polysaccharide peak from the first dimension SEC. A zoomed overlay can be seen in Figure 31, which shows the polysaccharide appearing in the second dimension RPLC chromatogram as the polysaccharide begins to be sampled from the first dimension. There is an expected 30 sec. delay in the appearance of the polysaccharide in the second dimension due to the time require for filling the sample loop and transferring. The appearance of the polysaccharide peak demonstrated successful transfer of the polysaccharide from the first dimension SEC to the second dimension RPLC for detection.

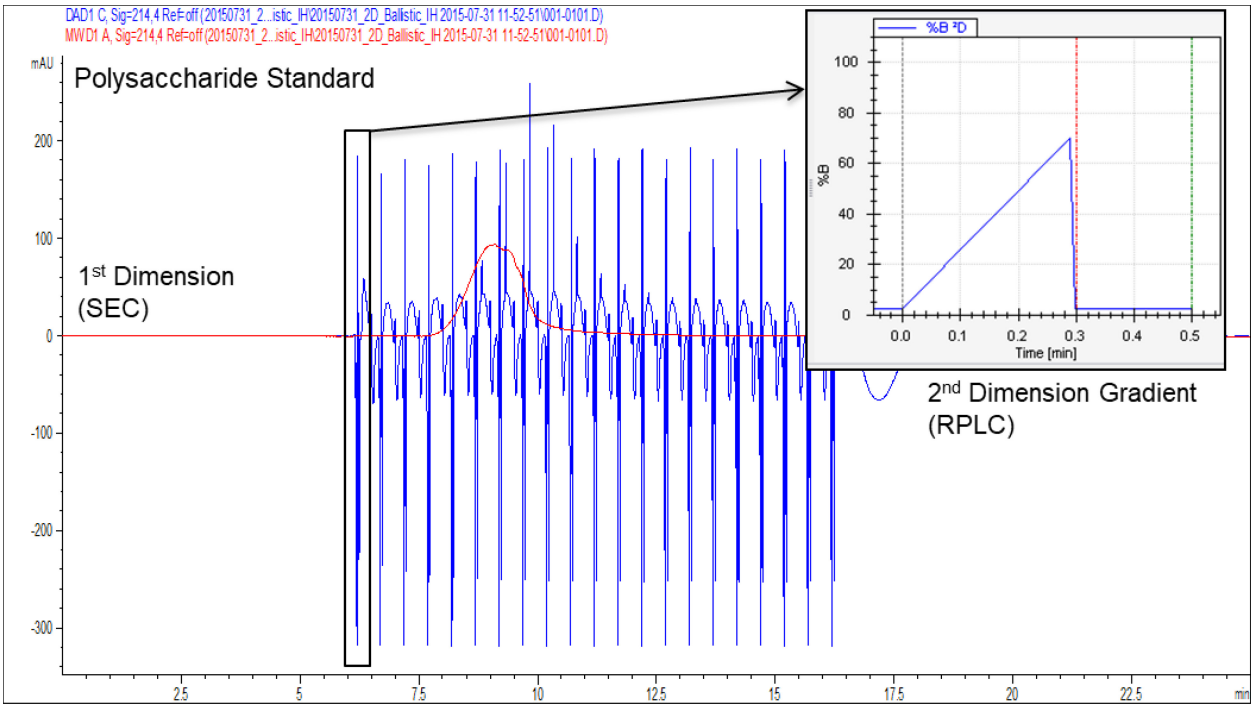

Figure 30. 2D-LC analysis of CP5 polysaccharide standard showing the first dimension SEC overlaid with the second dimension RPLC. The first dimension was sampled from 6 – 17 min. and subjected to further analysis by RPLC with a 0.5 min ballistic gradient.

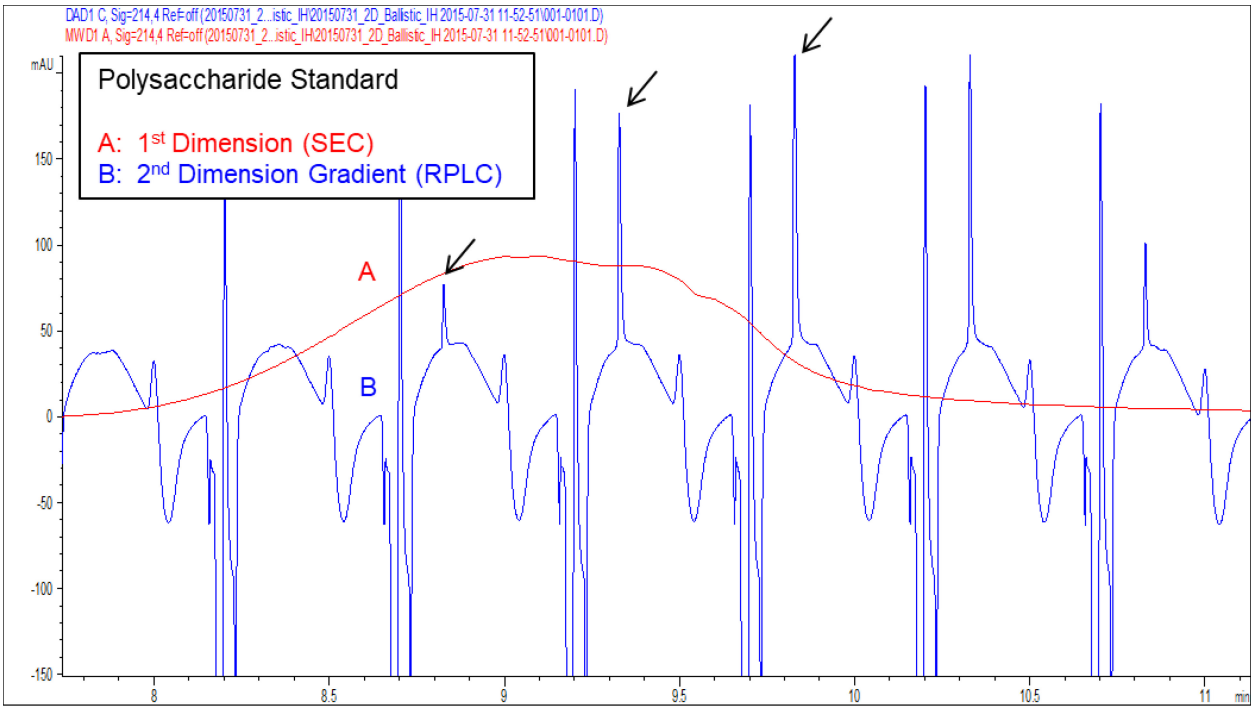

Figure 31. Zoomed overlay from 2D-LC analysis of CP5 polysaccharide standard showing the first dimension SEC (A) and the second dimension RPLC (B). The polysaccharide, indicated by arrows, observed in second dimension RPLC chromatogram as the polysaccharide peak is sampled from the first dimension.

Next, the analysis of a CP5-CRM$_{197}$ glycoconjugate spike sample was needed to demonstrate proof of concept for successful separation of all components of interest in a representative sample. The glycoconjugate spike sample was injected and analyzed similarly to the polysaccharide standard using the conditions listed in Table 10. The first dimension was again sampled from 6 – 17 min. transferring the glycoconjugate and carrier protein peaks to the second dimension and subjected to a 0.5 min. ballistic gradient for further analysis. The 2D-LC analysis of the glycoconjugate spike sample is shown in Figure 32 with the first dimension SEC (A) overlaid with the second dimension RPLC (B). A zoomed overlay can be seen in Figure 33, which shows the glycoconjugate and polysaccharide appearing in the second dimension RPLC chromatogram as the glycoconjugate peak begins to be sampled from the first dimension. The polysaccharide and glycoconjugate peaks are detected and fully separated in the second dimension. This demonstrated the feasibility and successful proof of concept of transferring the

polysaccharide and glycoconjugate peaks from the first dimension SEC to the second dimension RPLC for separation.

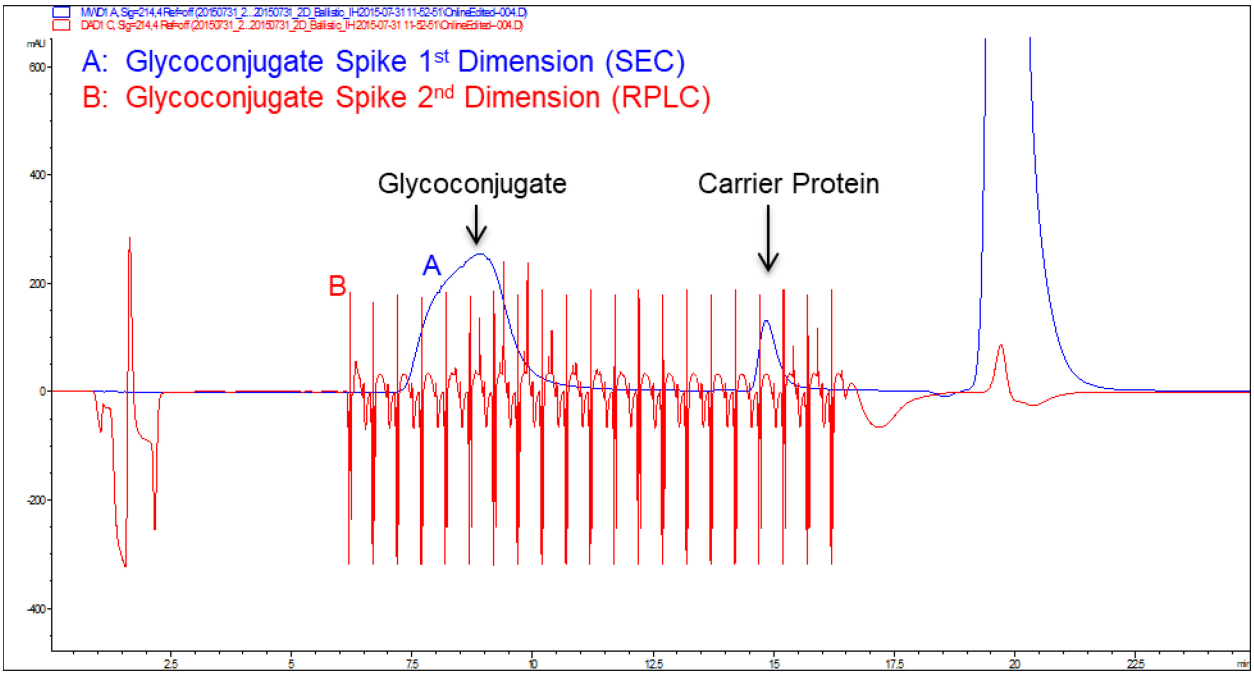

Figure 32. 2D-LC analysis of CP5-CRM$_{197}$ glycoconjugate spike sample showing the first dimension SEC (A) overlaid with the second dimension RPLC (B). First dimension sampled from ~6 – 16 min. and subjected to further analysis by RPLC with a 0.5 min ballistic gradient.

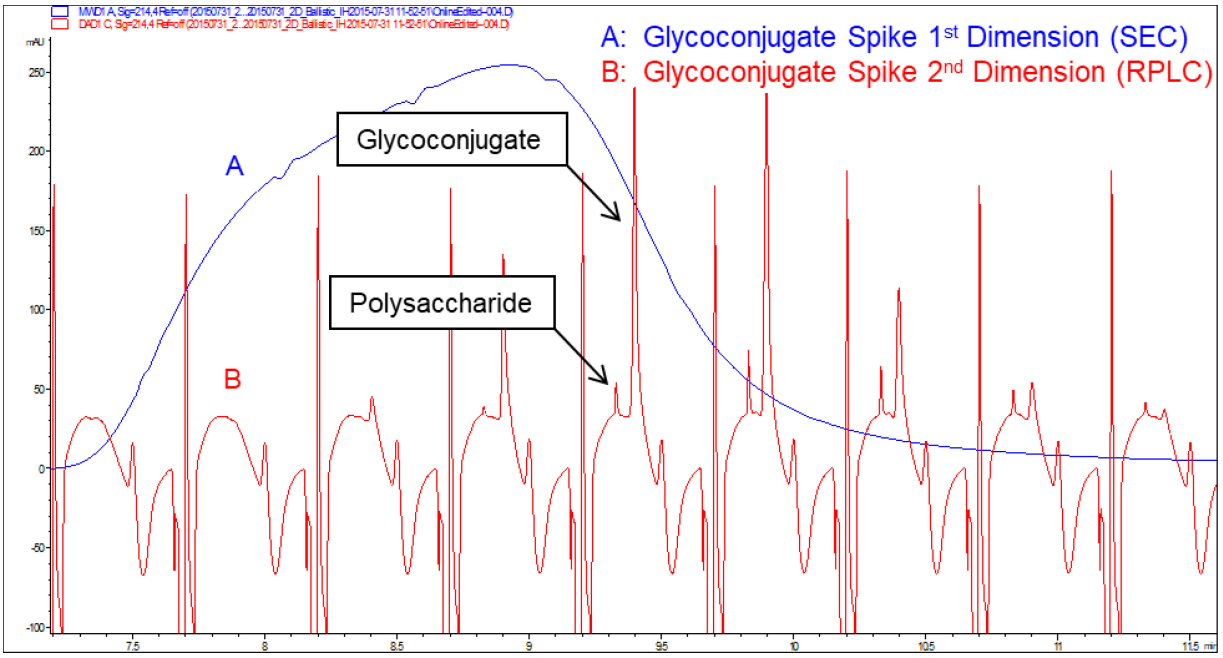

Figure 33. Zoomed overlay from 2D-LC analysis of CP5-CRM$_{197}$ glycoconjugate spike sample showing the first dimension SEC (A) and the second dimension RPLC (B). As the glycoconjugate peak is sampled from the first dimension, the polysaccharide and glycoconjugate peaks are further separated in the second dimension RPLC chromatogram.

Modification of 2D-LC Instrument Configuration and Method for Quantitation

The method conditions utilized during feasibility experiments in the previous section were not suitable for total quantitation of the polysaccharide in a glycoconjugate sample. To quantitate the amount of free polysaccharide, the entire peak would need to be transferred to the second dimension. Therefore, several critical changes were made to the 2D-LC method conditions used in the feasibility experiments. First, a larger, 2 mL loop was installed to capture the entire peak volume of the polysaccharide (approximately 0.45 mL). Next, the valve switching was changed to a single valve switch. For this application, the valve switches to position 2 and remains in the that position for remainder of the injection run time. Once the injection finishes, the valve returns to position 1 to prepare for the next injection. The polysaccharide and glycoconjugate peaks (co-eluting) enter the sample loop after the first dimension detector (flow path shown in Figure 29). First dimension SEC eluent immediately to the left of the switching time is

transferred to the second dimension as that volume has filled the sample loop. So, the valve switch time was targeted just after the polysaccharide peak. Lastly, the second dimension RPLC gradient would need modification for additional hold time at initial conditions. The purpose of gradient change was two-fold: equilibration of the second dimension baseline from the disturbance caused by the SEC mobile phase transfer and focusing of the polysaccharide peak at the head of the column to mitigate the effects of band broadening from the first dimension SEC separation. In addition, several method parameters reverted to the originally developed parameters in the previous 1D-sections, which include flow rates, RPLC column, mobile phase, and gradient program for RPLC. These were only changed during initial feasibility experiments to allow compatibility in a comprehensive 2D-LC format. Method parameters for quantitation 2D-LC are shown in Table 11.

Table 11: 2D-LC Instrumentation and Method Parameters for Quantitative 2D-LC Evaluation

Parameter	**1st Dimension – SEC**	**2nd Dimension - RPLC**
Pump	1260 Infinity Quaternary G5611A	1290 Infinity II Binary G7120A
Autosampler	1290 Infinity (G4226A)	
Detector	1260 Multi-wavelength (MWD) G1365D	1290 Infinity II Diode Array G7117B
Valve	1290 Infinity Valve Drive (8-port/2-position) G1170A	
Valve Loop volume	2000 µL	
Column	Waters Acquity UPLC BEH, 4.6 x 150 mm, 1.7 µm (186005225)	Agilent Zorbax SB 300 CN, 4.6 x 150 mm, 3.5 µm (863953-905)
Mobile Phase	30 mM Sodium Phosphate, 150 mM Sodium Chloride, pH 6.7	MPA: 0.10% TFA in Water MPB: 0.085% TFA in Isopropanol
Flow Rate	0.3 mL/min	0.5 mL/min
Gradient	Isocratic	Hold at 5% MPB – 5 min., then 5 – 70% MPB over 20 min., hold at 70% MPB for 5 min., return to 5% MPB and hold for 10 min.
Detection Mode	UV 280 nm	UV 214 nm
Column Temp.	30° C	40° C
Valve Configuration	See Figure 29	
Valve Loop volume	2.0 mL	
Valve Switch Time	4 min. (single valve switch)	
Injection Volume	10 µL	
Total Runtime	44 min (valve switch time plus second dimension run time)	

Polysaccharide and glycoconjugate samples were individually injected at 10 µL to target a 1 µg load and analyzed. The first dimension SEC chromatograms are shown in Figure 34 for the polysaccharide (A) and glycoconjugate (B). The line in Figure 34 at 4 min. indicates the time of switch to position 2 on the switching valve. As the sample loop volume is 2 mL and the flow rate is 0.3 mL/min., this allows for a sampling window of 6.7 min. For this separation, effectively all eluent to the left of the line was transferred. Though a portion of the glycoconjugate peak tail is not transferred to the second dimension, the peak of interest for quantitation in the second dimension is the polysaccharide, which is fully captured.

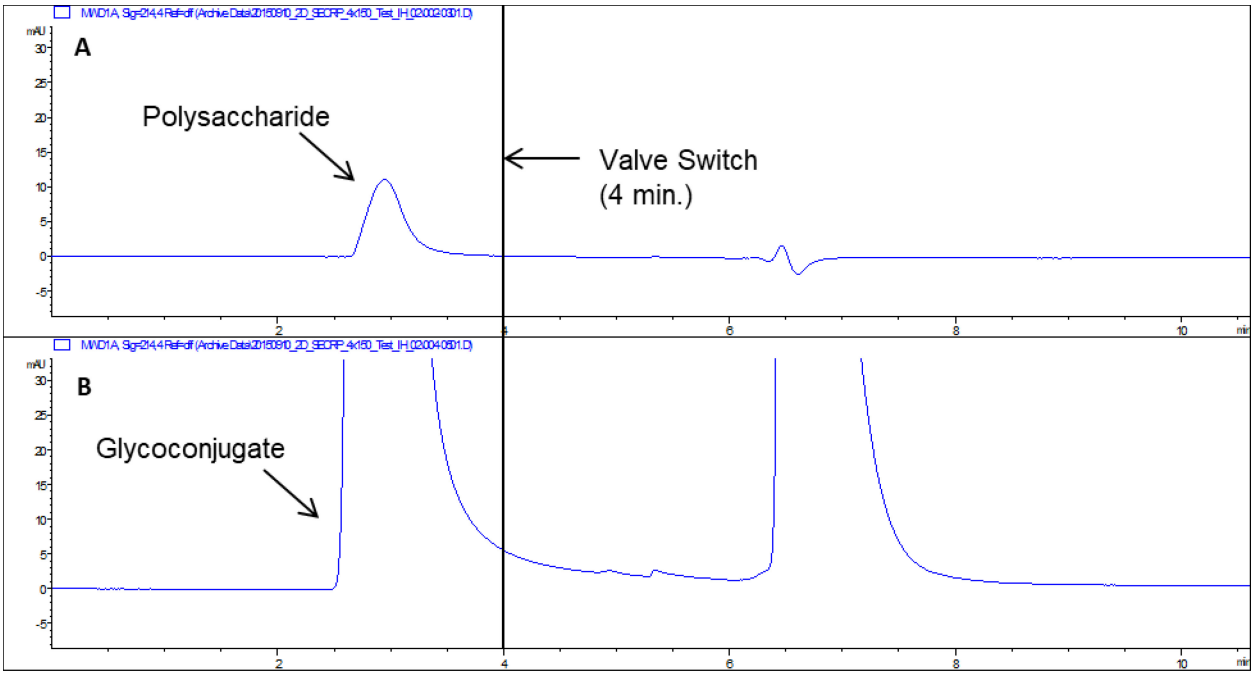

Figure 34 First dimension (SEC) of the 2D-LC analysis of samples A) polysaccharide B) glycoconjugate indiviually injected. Switching valve with a 2mL loop installed switched at 4 min. transferred all SEC eluent left of the line to the second dimension (RPLC) for further analysis.

Figure 35 shows an overlay of the subsequent second dimension RPLC separation of the individually injected glycoconjugate (A) and polysaccharide (B) samples. From approximately 9 – 14 min., a significant disturbance in the baseline is observed as the SEC eluent is transferred to the second dimension. The additional hold time at initial conditions prior to running the

elution gradient allows the SEC eluent to fully flow through the column and re-equilibration to establish a steady baseline. If the elution gradient were to begin immediately upon transferring, the chromatographic separation would almost certainly not be acceptable due to the disruption of the baseline and potentially analyte-stationary phase interaction. This hold-time also focuses the polysaccharide at the head of column to eliminate the band broadening from the first dimension which results in sharper peaks in the second dimension. As the elution gradient program is executed, the polysaccharide can be observed eluting at 22.5 min. in both samples. The glycoconjugate peak eluted later at 34 min. in the glycoconjugate sample.

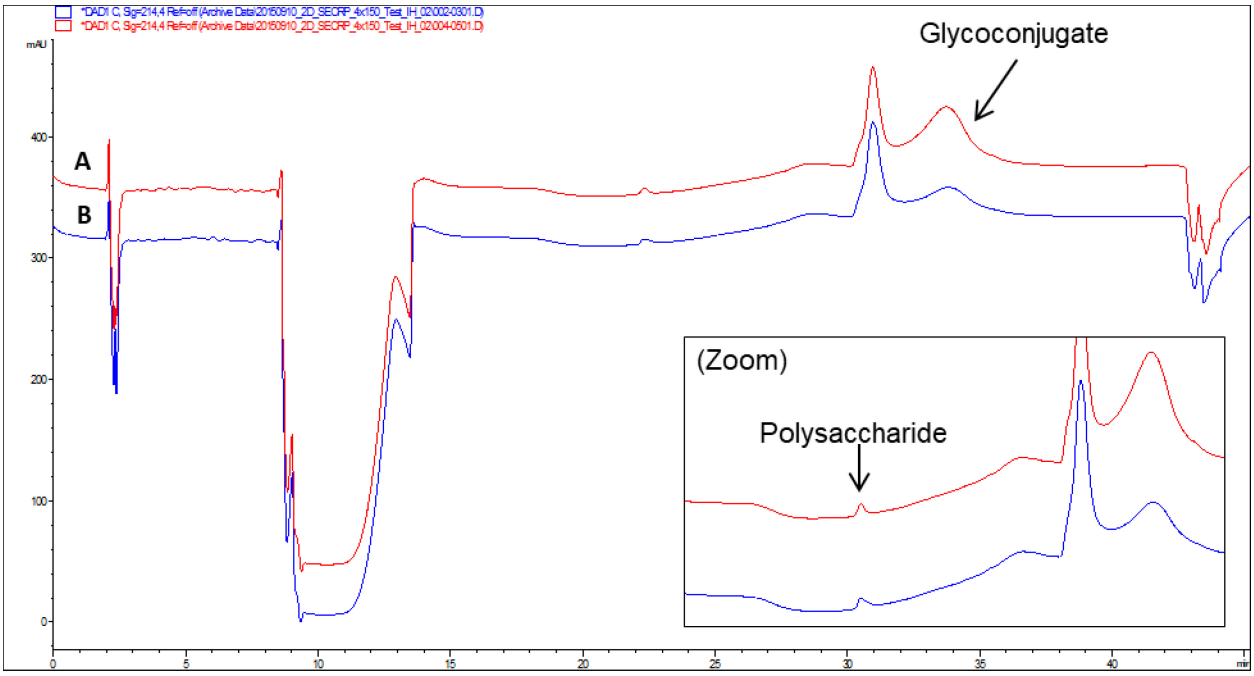

Figure 35 Second dimension (RPLC) of the 2D-LC analysis of samples A) glycoconjugate B) polysaccharide indiviually injected showing separation of polysaccharide peak in both samples. Glycoconjugate peak observed eluting at approximately 34 min in glycoconjugate sample injection.

This experiment demonstrated successful transfer of the polysaccharide peak from the first dimension SEC to the second dimension RPLC as well as adequate separation of the peaks of interest. Possible areas of improvement to the RPLC method were identified, such as reducing baseline drift, pressure, and analysis time. In addition, an experiment was performed where the

valve switch timing was changed from 4 to 5 min. to widen the snippet window. The purpose of this was to evaluate the robustness of the switching timing and its impact on polysaccharide peak recovery. The transfer timing was compared by calculating a percent recovery using the polysaccharide area counts from both conditions. The percent recovery was calculated to be 98.7% indicating consistent peak transfer when varying switching times. (Data not shown)

Optimized RPLC conditions and Linearity Evaluation in 2D-LC

Separating the peaks of interest in a quantitative 2D-LC format was successful. The next step in the 2D-LC development was evaluating linearity across an expected working range. Similarly, to the 1D method development, linearity was evaluated by analyzing carrier protein and polysaccharide standards with known concentrations at column loads ranging from 0.1 – 3.0 µg. Standards were diluted to 0.1 mg/mL and the injection volume was adjusted to achieve target column loads. The method conditions and parameters in Table 11 were utilized with the following exceptions: 900 µL valve loop, valve switch time of 5 min., RPLC flow rate increased to 1.0 mL/min, RPLC gradient used from Table 9, and the total run time was 25.5 min. These method modifications were made to address the possible method improvements stated in the previous section. The chromatograms of both dimension separations can be seen in Figure 36 along with example integrations that were applied to the individual carrier protein and polysaccharide standards.

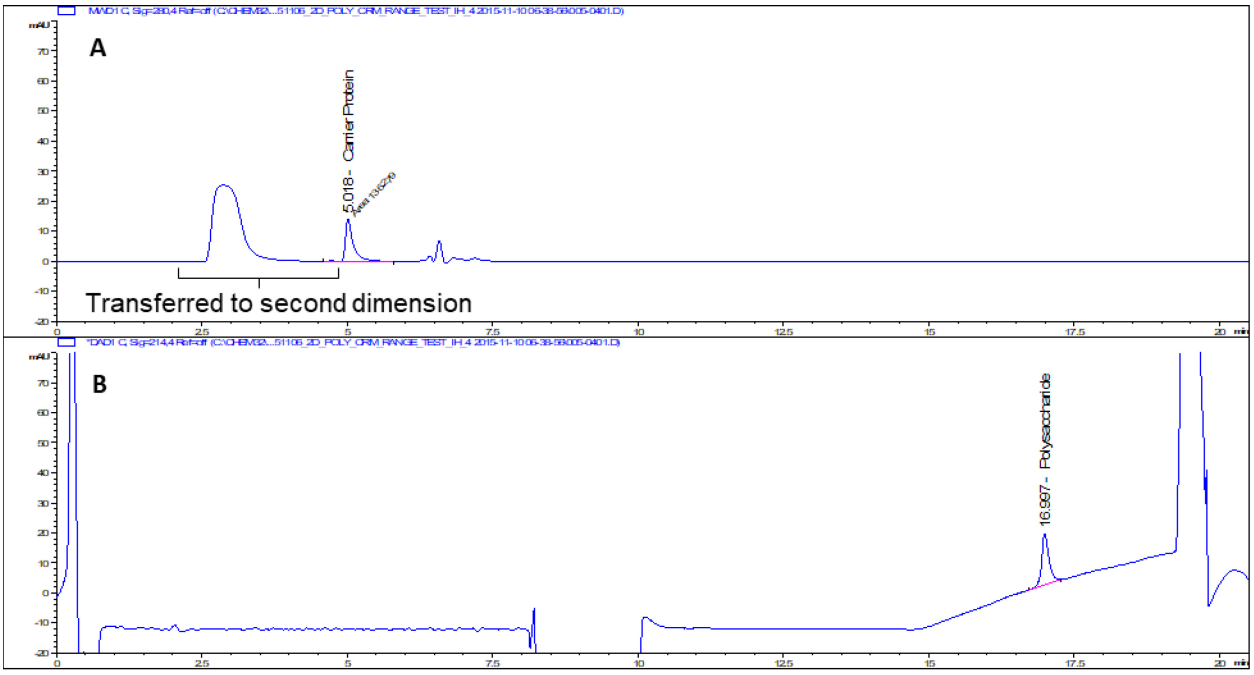

Figure 36 2D-LC analysis of glycoconjugate spike sample showing zoomed chromatograms from the separation of A) carrier protein in the first dimension by SEC and B) polysaccharide in the second dimension by RPLC.

Significant improvement in the baseline and overall peak shape was observed in the second dimension for the separation of polysaccharide. In addition, the total analysis time was reduced by 45%, saving 20 min. per injection. Therefore, the changes made have resulted in an overall improved method.

Linearity of both carrier protein and polysaccharide peaks was evaluated using column loads ranging from 0.1 to 3.0 µg. All chromatograms were integrated, a linear regression was performed using peak area vs. µg column load, and response factors were calculated. A summary of these data is shown in Table 12. Response factor plots were generated with reference bars at 90 and 110% of the average response factor across the range for reference. The linear line fit and response factor plots are shown in Figure 37.

Table 12 Summary of column load, area count, and calculated response factors from the separation of the carrier protein in the first dimension (SEC) and polysaccharide in the second dimension (RPLC)

	1D SEC - Carrier Protein		2D RPLC - Polysaccharide	
Column Load (µg)	Area 280 nm (µV*sec)	Response Factor (response/load)	Area 214 nm (µV*sec)	Response Factor (response/load)
0.1	13.9	139.4	3.0	30.0
0.5	69.1	138.2	26.7	53.4
1.0	138.2	138.2	59.3	59.3
1.5	211.2	140.8	97.3	64.8
2.0	285.7	142.9	129.1	64.6
2.5	360.2	144.1	175.5	70.2
3.0	435.2	145.1	219.3	73.1

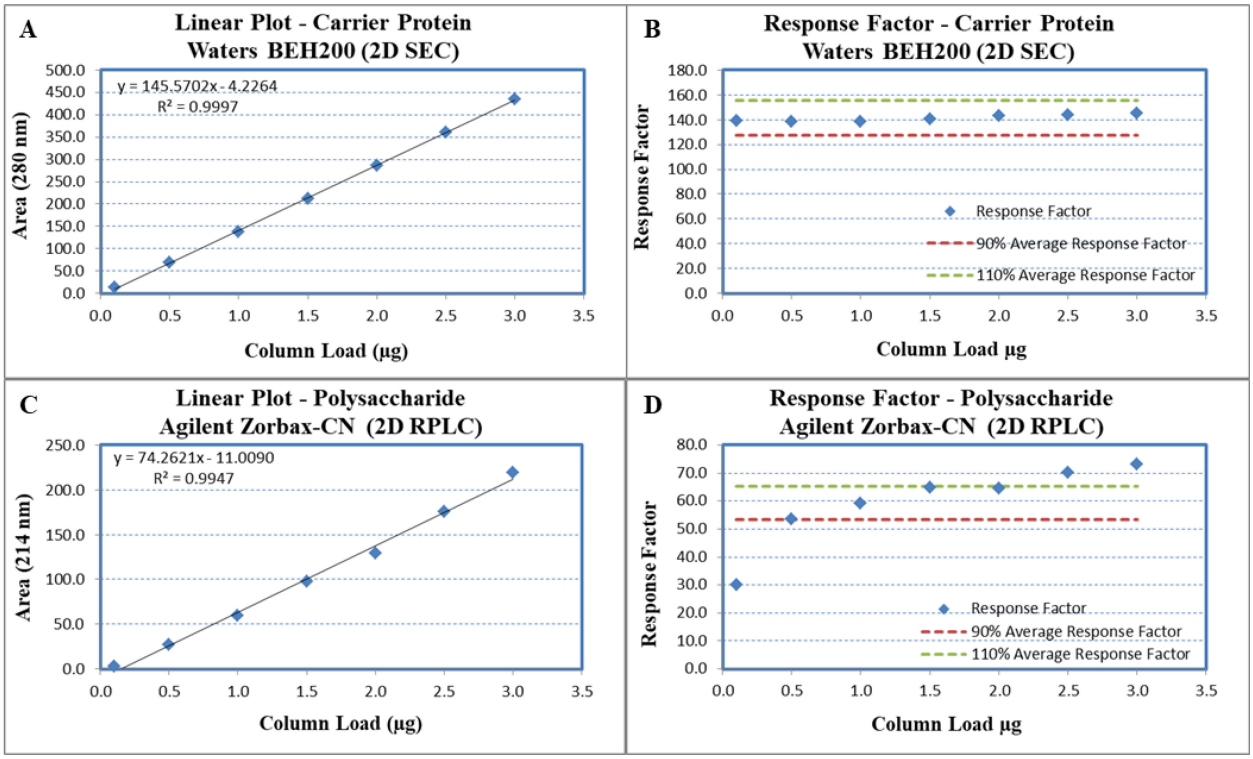

Figure 37 A) Carrier protein linear plot and B) response factor plot from first dimension SEC. C) Polysaccharide linear plot and D) response factor plot from the second dimension RPLC.

No significant bias was observed and appeared linear across the range tested for the carrier protein. However, a significant trend was observed for the polysaccharide. Though the coefficient of determination (R^2) is greater than 0.99, visual inspection of the line fit plot shows deviation from the regression line at several points across the range. The response factors calculated and plotted show a significant increasing trend with column load indicating the

presence of a bias and non-linearity across the range. Therefore, the current second dimension method was not suitable for quantitation. Upon reviewing the 1D RPLC linearity data, the trend was indeed present, but to a lesser degree, initially this was thought to be an effect of approaching the quantitation limit. However, this bias is shown to be real and exacerbated in the 2D-LC format. With the consistency of this bias being present in both 1D and 2D-LC formats, the non-linearity and poor recovery at the lower concentrations could be due to weak interactions of the polysaccharide with the column stationary phase. Additional columns were explored to find an alternative for this application.

The first column evaluated was an Agilent Poroshell SB-300 C3, 5 µm, 2.1 x 75 mm (660750-909). This utilizes the same silica and "StableBond" manufacturing technique used in the previous cyano column, but with a fused-core particle, or otherwise termed superficially porous particle (SPP). This particle has a solid core of silica with a thin layer of porous silica bonded to it. This creates a smaller diffusion distance for the analyte and allows for column efficiencies like that of sub-2 µm particles with significantly less back pressure and analysis time [21]. Columns packed with superficially porous particles (SPP) typically utilize a higher flow rates to achieve high efficiencies due to the increased size of the particle. This required new method parameters, which are shown in Table 13. The mobile phase B composition was modified to incorporate acetonitrile and reduce the backpressure from the isopropanol.

Table 13. RPLC Method Conditions for Free Polysaccharide using Agilent Poroshell

Parameter	Setting
Column	Agilent Poroshell SB-300 C3, 5 µm, 2.1 x 75 mm (660750-909)
Mobile Phase A (MPA)	0.10% TFA in Water
Mobile Phase B (MPB)	0.085% TFA in IPA:ACN:H20 (70:20:10)
Flow Rate	1.2 mL/min
Column Temperature	40° C
Elution Gradient Program	Hold at 5% MPB – 2.5 min., then 5 – 80% MPB for 10 min., hold at 80% MPB for 3.5 min., return to 5% MPB and hold for 4 min.
UV Wavelength	214 nm
Run Time	20 min.

The polysaccharide standard (0.1 mg/mL), glycoconjugate spike (~20% free polysaccharide), and water were all individually injected at 10, 5, and 20 µL, respectively. An overlay of the three injections can be seen in Figure 38, where the overall separation of the polysaccharide and glycoconjugate peaks is accomplished with acceptable chromatography. Elution time, flushing, and equilibration times all appeared more than adequate, and could potentially be optimized by shortening the timing of the respective steps. An interesting observation was the slight difference in peak width of the polysaccharide peak between the polysaccharide standard and glycoconjugate spike sample (peaks are of similar area count). This broadening of the free polysaccharide is thought to be due to the activation of the polysaccharide prior to conjugation causing heterogeneity of species for un-conjugated polysaccharide.

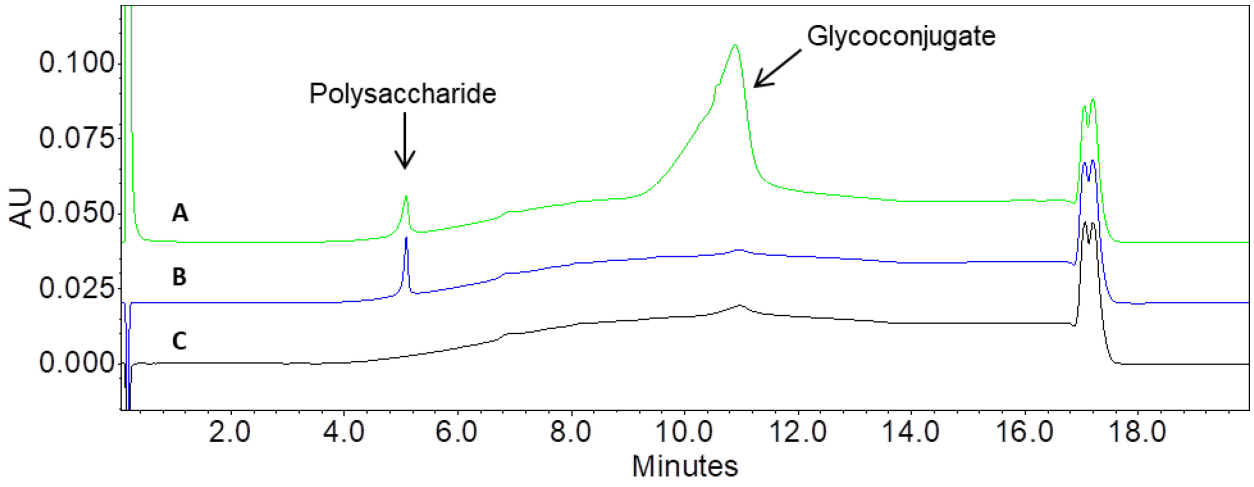

Figure 38 1D RPLC analysis of samples A) glycoconjugate spike B) polysaccharide standard C) water indiviually injected showing separation of polysaccharide peak in non-placebo samples. Glycoconjugate peak observed eluting at approximately 11 min. in glycoconjugate spike sample injection.

Linearity of the polysaccharide peak using the polysaccharide was evaluated using a column load ranging from 0.1 to 3.0 µg (achieved by variable injection volume). All chromatograms were integrated, a linear regression was performed using peak area vs. µg column load, and response factors were calculated. A summary of these data is shown in Table 14. Response factor plots were generated with limit bars at 90 and 110% of the average response factor across the range for reference. The linear regression fit of the data and response factor plots are shown in Figure 39. Visual inspection of the linear regression plot showed the response to be linear with an R^2 of 1.000. The response factor plot showed no significant bias or trend across the range tested. Overall, the Poroshell SB-C3 column showed much improved linearity for the polysaccharide peak compared to the Zorbax SB-CN.

Table 14 Summary of column load, area count, and calculated response factors from separation of polysaccharide by 1D RPLC.

Column Load (µg)	Area 214 nm (µV*sec)	Response Factor (response/load)
0.1	10700	107000
0.5	55670	111340
1.0	114777	114777
1.5	170344	113563
2.0	227844	113922
2.5	286295	114518
3.0	344286	114762

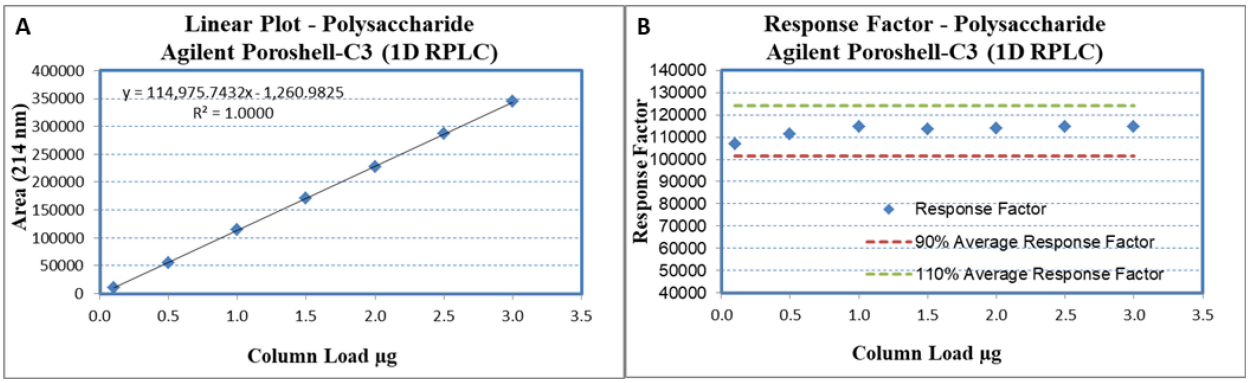

Figure 39 A) Polysaccharide linear plot and B) response factor plot from 1D RPLC analysis.

Note, Waters X-Bridge BEH C4, 3.5 µm, 300Å, 2.1 x 150 mm (186004500) and Phenomenex Jupiter C4, 5 µm, 300Å, 2 x 150 mm (00F-4167-B0) colmns with fully porous particles were also evaluated (data not shown). The Phenomenex Jupiter C4 showed a trend of increasing response factor similar to the Agilent Zorbax SB-CN column. The Waters BEH C4 column showed acceptable linearity, but slight interference was observed in the region of the polysaccharide peak, which could lead to potential specificity issues. Therefore, the Agilent Poroshell C3 column was selected to be utilized as the second dimension RPLC column.

3.6.2. Robustness Challenges with Glycoconjugate Analysis and 2D-LC Instrument Parameters

The majority of experiments have been executed focusing on the analysis of purified reference standards of the individual glycoconjugate components. Glycoconjugate samples had not been

previously analyzed with high replication or a broad range of free protein and/or polysaccharide. However, as experiments were performed where multiple glycoconjugate and glycoconjugate spike samples were analyzed, issues in both dimensions arose. In addition, the impact of valve switching with the 2D-LC method parameters were not yet fully understood. These issues were critical to overcome in order to develop a robust method.

Valve-Switch Timing in First Dimension (Pressure Disturbance)

The timing of the valve switch required careful consideration to ensure full transfer of the polysaccharide peak from the first dimesion to the second dimesion. As first dimension SEC eluent immediately to the left of the valve switch time is transferred to the second dimension, programming the time of this switch too close to the peak of interest can result in a loss of recovery in the second dimension. During the initial feasability experiments, the valve switch time was set at 4 minutes in the first dimension SEC analysis, just after the retention time of polysaccharide peak of 3 minutes (shown in Figure 34). Robustness of this valve switch timing was evaluated by moving the time to 5 minutes to ensure no loss of the polysaccharide peak. The second dimension polysaccharide peak was analyzed and integrated using a 4 and 5 minute switch time. The area counts were then used to calculate a percent recovery of 98.7% (96.1 mAU with 4 minute switch; 99.4 mAU with 5 minute switch). This indicated no significant impact in recovery using a valve switch time of 4 and 5 minutes. A switching time of 4.5 minutes was then carried forward in the 2D-LC method.

However, after implementing the 4.5 minute valve switch, an interferring peak was noticed in the first dimesion UV signal of a water (blank) injection near the timing of the valve switch. This peak interfered with the carrier protein peak and could impact peak area recovery. Figure 40

shows the UV (280nm) and pressure signals of a water and carrier protein standard from the first dimension SEC analysis. The pressure channels also showed a pressure spike matching the time of the distrubance in the UV signal. The disturbances in these signals all occurring near the time of the valve switch indicated the valve switch was likely the cause.

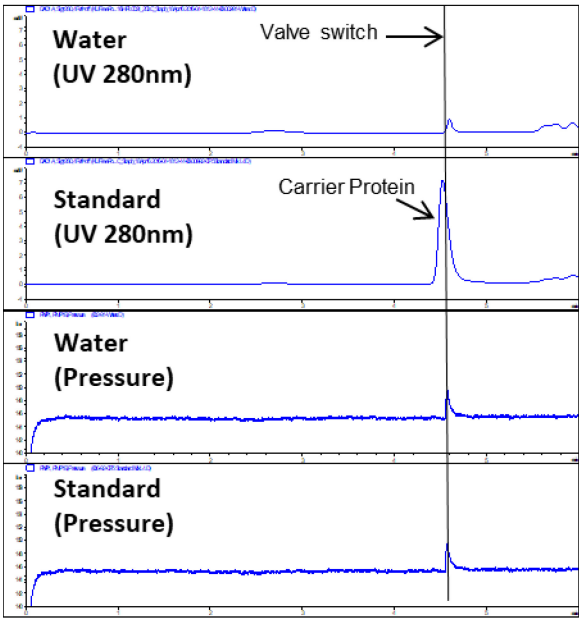

Figure 40 2D analysis chromatograms and pressure traces of water and carrier protein standard from the first dimension SEC. The valve switch, indicated with line at 4.5 min, disturbs the baseline in the water injection in the UV signal as well as pressure traces for the water and carrier protein.

To evaluate this further, the valve switch time was changed to 5.5 minutes, after the retention time of the carrier protein peak. This would move the interference away from the carrier protein peak and effectively transfer the polysaccharide, glycoconjugate, and carrier protein peak to the second dimension. This would still allow for quantitation of free carrier protein in the first dimension and free polysaccharide in the second dimension. Figure 41 shows the UV (280nm) and pressure signals of a water and carrier protein standard from the first dimension SEC analysis with a valve switch time of 5.5 minutes.

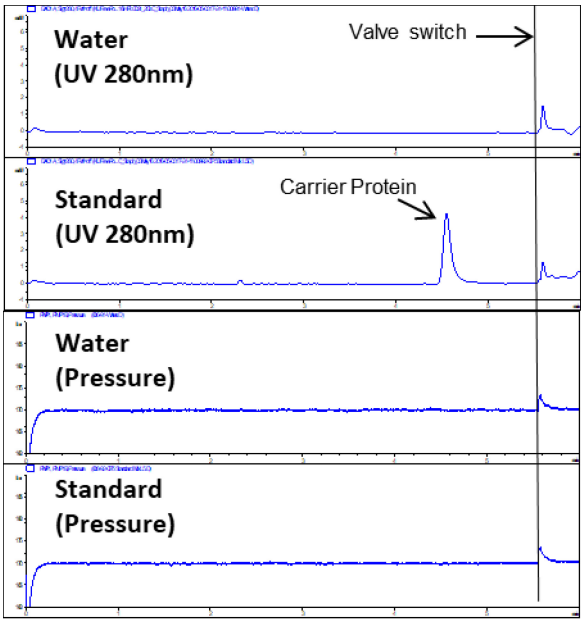

Figure 41 2D analysis chromatograms and pressure traces of water and carrier protein standard from first dimension SEC. The valve switch, indicated with line at 5.5 min, disturbance observed in all traces does not interfere with the carrier protein peak.

Tthe disturbance is observed in all signals, but now near 5.5 minutes, confirming the cause to be the valve switch. The later switching time mitigated the interference with the carrier protein. As the flow path of the switching valve is directly in line with the first dimension detector, the pressure ripple from the valve switch likely caused the disturbance in the first dimension isocratic separation. The interferring peak was integrated and the area was compared to that of the carrier protein peak (0.5 µg column load) in the same chromatogram. The percent contribution of the interferring peak calculated to be 9.5% (2.6/27.4*100 = 9.5%). For a quantitative residual impurity assay, low limits of quantitation are desired, and an interference of that percentange would be problematic. The modification of the valve switching time mitigated the interference issues.

Impact of Sample Filtration on SEC Column Performance

Glycoconjugate spike samples were analyzed as a pre-qualification experiment to screen linearity of the free carrier protein using representative samples with a broad range of free carrier protein and polysaccharide. CP5 and CP8-CRM_{197} glycoconjugate samples were prepared by spiking the respective glycoconjugate sample with carrier protein and polysaccharide standards. The glycoconjugate final volumes and concentration (1.0 mg/ml) were held constant by varying the amounts of carrier protein, polysaccharide, and placebo to achieve a range of 1 – 20% free carrier protein and 2 – 44% free polysaccharide. A sequence containing 28 injections of spike samples (14 of each serotype) was programmed. Method conditions from Table 7 were used with the exception that EDTA was omitted from the mobile phase and the samples were injected at 15 µL. Chromatography appeared as expected until the sequence neared the end of the run. Figure 42 illustrates chromatograms from the analysis of glycoconjugate spike samples by SEC, where (A) is an expected chromatographic profile and (B) is the profile observed after the analysis of 10 or more samples. Significant performance loss was observed, such as peak broadening, in the carrier protein peak and throughout chromatogram, resulting in decreased recovery of carrier protein.

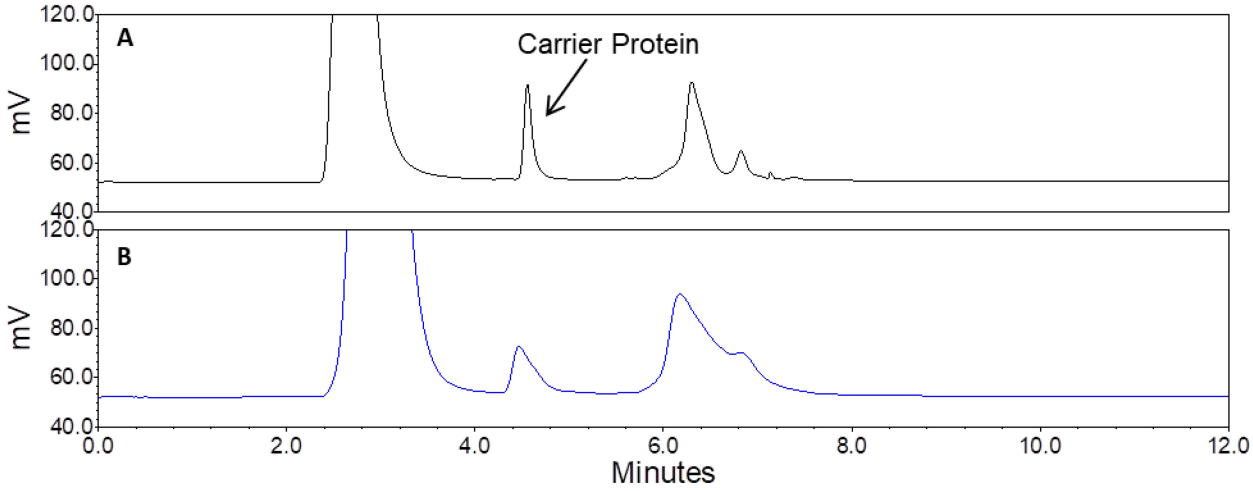

Figure 42 1D SEC analysis of CP8-CRM$_{197}$ glycoconjugate spike samples for free carrier protein. A) Representative chromatogram and profile of the glycoconjugate spike sample observed. B) Chromatogram from the analysis of spike samples showing broadening of carrier protein peak after analyzing a large number of samples.

After the loss of performance was observed, the pressure signals were examined to assess any system issues. Pressure traces of twelve glycoconjugate samples over the course of the sequence were overlaid and can be seen in Figure 43. An increasing trend in pressure is clearly observed, which resulted in an overall increase of approximately 12 bar. Theses subtle increases were likely occuring in previous development experiments, but were undetectable and a non-issue when analyzing low numbers of glycoconjugate samples. However, this increase becomes more problematic as the sample load increases and pressure increases accumulate. Due to the conjugation process, glyconconjuate samples are large in molecular weight with a heterogeneous distribution of molecular sizes. This results in increase polydispersity of molecular weight species. Considering the small inlet frit porosity of the Waters BEH200 column is 0.2 µm (obtained from Waters), there is concern of clogging the inlet frit with large soluble species or non-visible insoluble particles. Adsorption to the frit is also a possibility. The disruption of analyte flow path through the inlet frit could be the cause of peak splitting and pressure increases observed in the SEC analysis.

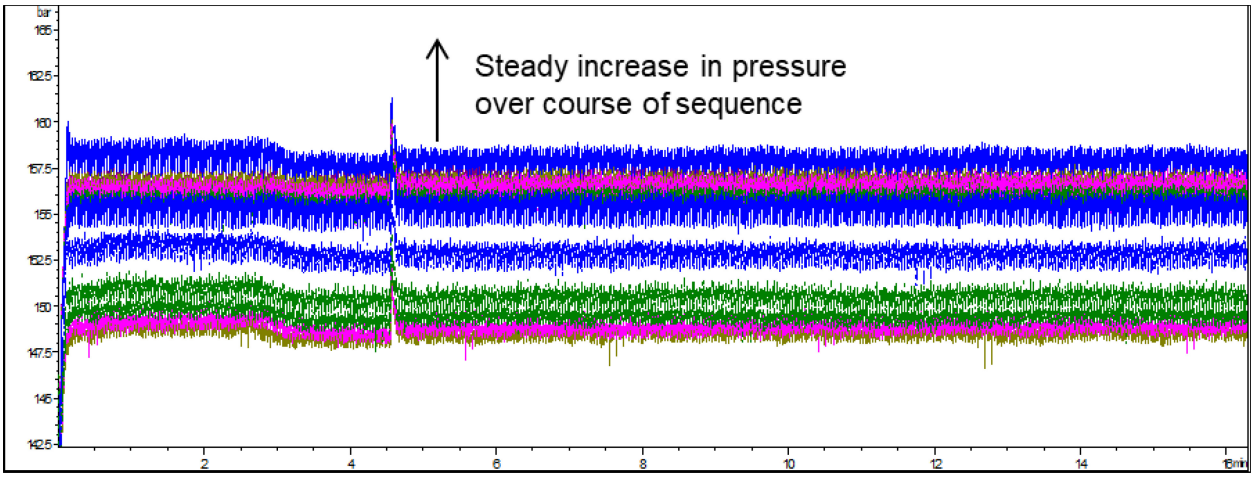

Figure 43 Overlay of pressure traces from the SEC analysis of 12 glycoconjugate spike samples in the same sequence. An increase in pressure was observed with each sequential injection of sample throughout the course of the sequence.

A filtration step was implemented to address the potential inlet clogging and mitigate the pressure increase in the first dimension SEC. The intent was to remove the large, soluble glycoconjugate species, and though no obvious visible insoluble particulates were observed, these would also be removed. A 0.2 µm syringe filtration device (part number 4454) manufactured by PALL (Port Washington, NY) was chosen as this device accommodates smaller volumes to minimize sample consumption. The sample preparation consisted of transferring 0.5 mL of sample volume into a 1 mL sterile syringe. The filtration device was then attached, and the sample was expelled through the filtration device into a glass HPLC vial. The HPLC vial was then placed into the autosampler and directly analyzed for free carrier protein by SEC and free polysaccharide by RPLC in 1D formats. For both SEC and RPLC, pressure was monitored and peak area with and without filtration was used to evaluate recovery from the filtration device. To prevent further clogging in the SEC, a previous analysis of the unfiltered glycoconjugate spike sample was used for evaluation of carrier protein recovery. This used a larger injection volume of 10 µL, whereas the filtered sample was injected at 5 µL. Therefore, a recovery of 50% was targeted for the carrier protein peak. A summary of the peak areas and

recoveries of the carrier protein (SEC) and polysaccharide (RPLC) peaks is shown in Table 15. Both components showed acceptable recovery within 10% to give confidence no significant analyte was lost during the sample filtration step.

Table 15 Recovery evaluation of carrier protein and polysaccharide in a glycoconjugate spike sample after sample filtration

	Carrier Protein (SEC)	Polysaccharide (RPLC)
Filtered Area	4497	66849
Unfiltered Area	9791	67498
Recovery (%)	45.9*	99.0

*Targeted 50% recovery due to injection volume of filtered sample half of un-filtered

In addition, the repeat analysis of spike samples was performed to challenge the robustness of the filtration step. Again, the pressure traces of twelve CP5-CRM_{197} and CP8-CRM_{197} glycoconjugate samples over the course of the sequence were overlaid, which can be seen in Figure 44. No obvious trend in pressure is observed and the pressure appeared to stable within ±1 bar. Note, the overall pressure is higher than the previous pressure overlay (Figure 43). This was attributed to plumbing changes made between sequences. Also, chromatographic profiles for all spike samples were as expected over the course of the sequence.

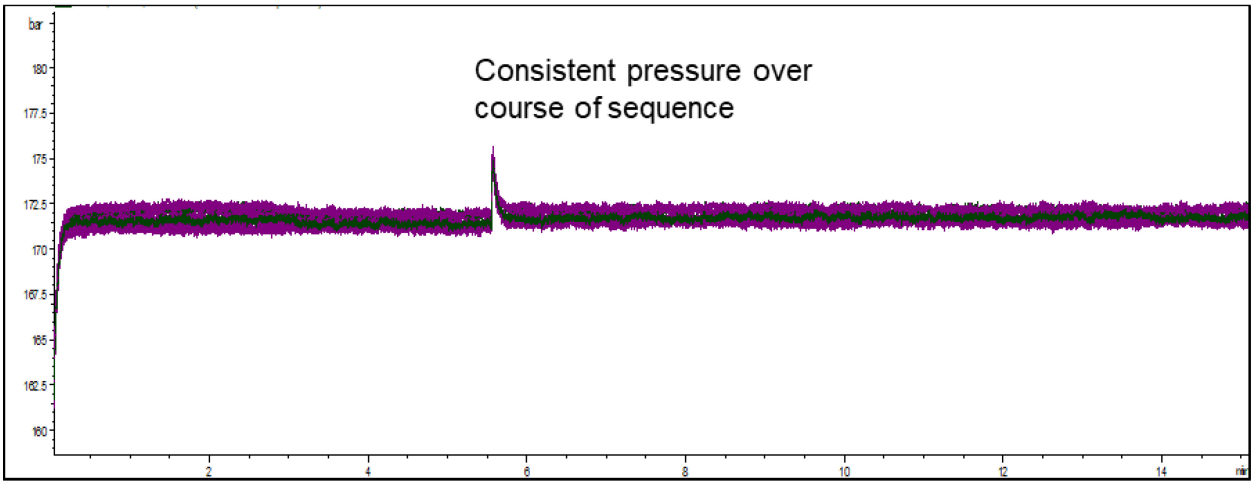

Figure 44 Overlay of pressure traces from the SEC analysis of 12 glycoconjugate spike samples in the same sequence. After filtration of sample prior to analysis, no significnant change in pressure was observed throughout the course of the sequence.

To investigate the impact of the filtration and the cause of performance improvements, non-filtered and filtered glycoconjugate spikes samples were analyzed by SEC-MALLS (multi-angle laser light scattering) for molecular weight analysis. SEC-MALLS has been routinely utilized for the analysis of average molecular weight of glycoconjugate vaccines [22]. Analysis was performed on an Agilent 1200 coupled to a Wyatt (Santa Barbara, CA) Optilab rEX Refractive Index (RI) and DAWN HELEOS MALLS detector. The RI of the mobile phase was measured by the RI detector at 30° C. The determination of the molecular weight was performed by the ASTRA software. The separation was performed on a TSK-gel mixed bed GMPWxl 300x7.5 mm GPC column (part 08025) held at 30° C. The isocratic flow rate was 0.5 mL/min using a mobile phase consisting of sodium chloride, sodium phosphate, EDTA, pH 6.7 with acetonitrile. Samples were diluted to 1 mg/mL in water and injected to target a 40 µg column load. The average molecular weight results for non-filtered and filtered glycoconjugate spike samples are summarized in Table 16. The results did not show any obvious trend in molecular weight outside of normal variability for CP5-CRM_{197} or CP8-CRM_{197} after filtration. If large soluble molecular species are being removed from the samples in the filtration step, they are likely a small percentage of the overall distribution. The resulting increased column performance and robustness after implementing the sample filtration could be more likely due to the removal of non-visible insoluble species or particles. However, this will need further experimentation to confirm.

Table 16 Molecular weight analysis by SEC-MALLS for non-filtered and filtered glycoconjugate spike samples

Sample	Non-Filtered Average Molecular Weight (kDa)	Filtered Average Molecular Weight (kDa)
CP5-CRM_{197} Low	2101	2031
CP5-CRM_{197} Mid	2043	1983
CP5-CRM_{197} High	1922	1881

CP8-CRM_{197} Low	1009	997
CP8-CRM_{197} Mid	977	967
CP8-CRM_{197} High	902	896

In summary, the implementation of the sample filtration step successfully mitigated the pressure and performance issues associated with the likely clogging of the column inlet frit.

Cleaning Step for RPLC

Several challenges were encountered during the development of the 1D method for free polysaccharide by RPLC. One was due to the non-linearity of the polysaccharide standard previously discussed in section 3.6.1. This issue was remedied by a column change with a different stationary phase. After the column change, robustness was evaluated by analyzing glycoconjugate samples with increased replication. Method conditions from Table 13 were utilized as this experiment was performed following the standard linearity experiment using the Agilent Poroshell C3 column described in 3.6.1. A water (blank) was analyzed followed by triplicate injection of a glycoconjugate sample at 5 µL. An overlay of the pressure and UV (214nm) signals are shown in Figure 45 and Figure 46, respectively. A significant increase in pressure can be observed with the first injection of glycoconjugate sample, and even greater increases with each sequential injection. This increase is problematic and will eventually lead to the HPLC system shutting down due to over-pressuring. Due to the hydrophobic properties of the glycoconjugate, the pressure increases are theorized to be caused by the inability to elute the conjugate at 80% organic mobile phase, or the glycoconjugate could be precipitating out of solution onto the column during the wash steps. Interestingly though, the peak shape and recovery of the polysaccharide in the UV signal appear to be unaffected by the pressure increases.

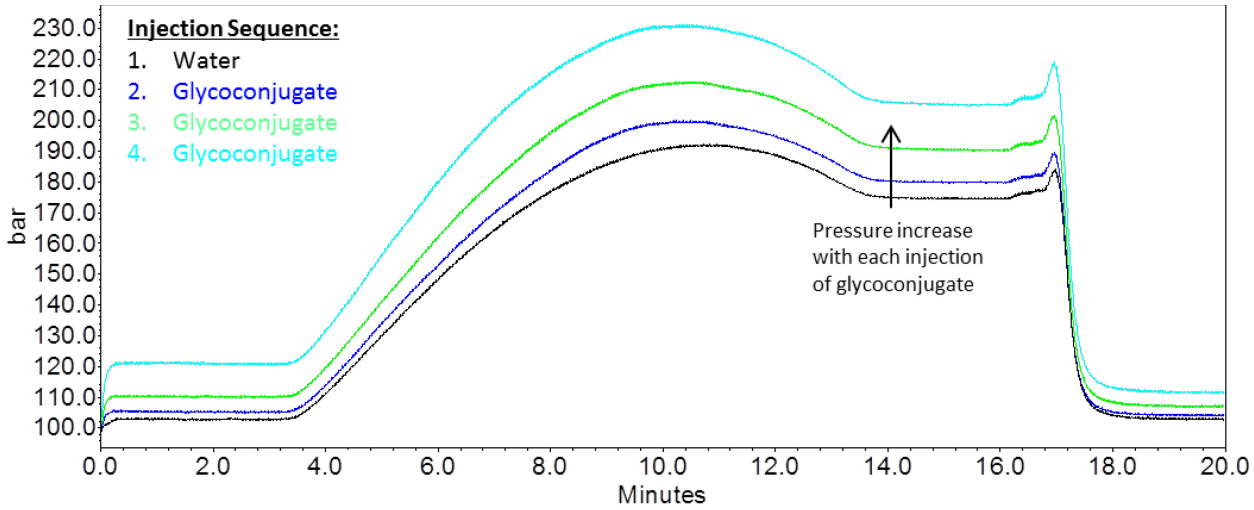

Figure 45 Pressure signal overlay of water (blank) followed by sequential glycoconjugate samples. Significant increase in pressure observed after each glycoconjugate injection.

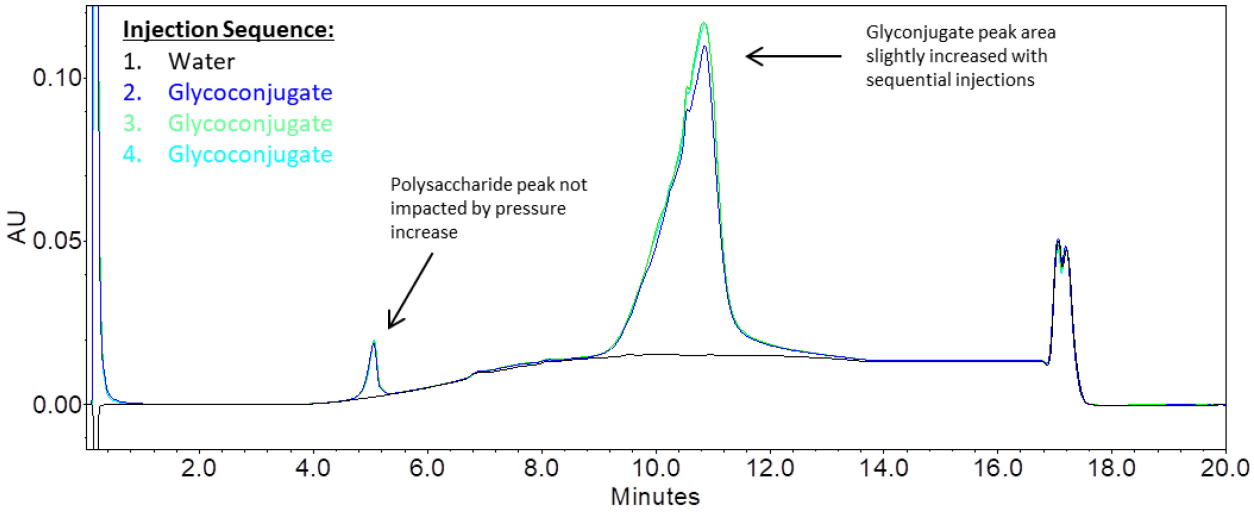

Figure 46 UV (214 nm) signal overlay of water (blank) followed by sequential glycoconjugate samples (same injections shown in Figure 45). Increasing pressure showed no observable impact on polysaccharide peak shape or recovery, whereas the glycoconjugate peak showed slightly increasing area counts indicating carry-over.

As mentioned, the insolubility of the glycoconjugates in organic solvents was considered to be a potential cause of the pressure increase. From prior experiences, glycoconjugates have been shown to be insoluble at high percentages of organic solvents, which could potentially cause on-column precipitation resulting in pressure increases and decreasing column performance. To evaluate solubility, glycoconjugate sample was spiked into various organic mixtures ranging from pure water to 100% acetonitrile (20 µL glycoconjugate into 1000 µL solvent mixture).

Precipitation could be clearly observed at 80% organic and above with the presence of white, amorphous particles (image shown in Figure 47). The gradient in Figure 45 and Figure 46 utilized 80% mobile phase B as the washing step.

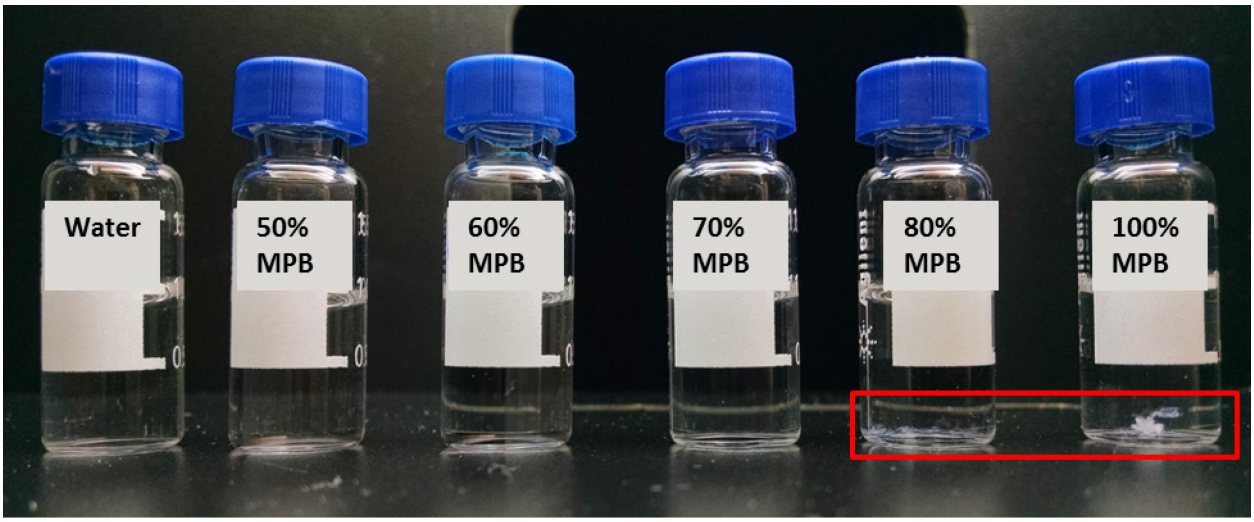

Figure 47: 20 µL of a glycoconjugate sample spiked into water and a MPA (0.1% TFA in water) mixed with increasing concentration of MPB (0.085% TFA in ACN concentration). Precipitation observed at 80% MPB and greater.

Experiments were then performed to evaluate lower % MPB for elution from the column, which reduced the %MPB from 80 to 70% (data not shown). However, reducing the maximum % MPB still resulted in the same pressure issue observed in Figure 45. Even though reducing the amount of organic in the mobile phase was not a solution to the problem, useful information on method limitations were still obtained. High organic concentrations should be avoided to prevent on-column precipitation if possible.

The cause of the consistent pressure increase was further thought to be caused by the inability to elute the glycoconjugate due to strong binding to the stationary phase. Compounds such as guanidine are capable of solubilizing protein and are often recommended in column cleaning procedures. As the peak of interest is the polysaccharide and the peak performance does not

appear to be negatively impacted by the pressure increase, implementing a column cleaning step was feasible. A series of six injections of a glycoconjugate sample was injected at 5 µL followed by an injection of 8 M guanidine hydrochloride (GuHCl) at 50 µL and then water at 5 µL. Pressures traces from these injections are shown in Figure 48. Pressure increased as expected with each glycoconjugate injection as seen in Figure 48 (A). However, Figure 48 (B) shows with one injection of 8 M GuHCl, pressure returned to initial operating levels prior to any glycoconjugate injection. This was an acceptable to addition into the method as the polysaccharide peak appears unaffected by the pressure increase and bracketing every six injections of glycoconjugate with a cleaning step does not greatly increase overall run time of a sample sequence.

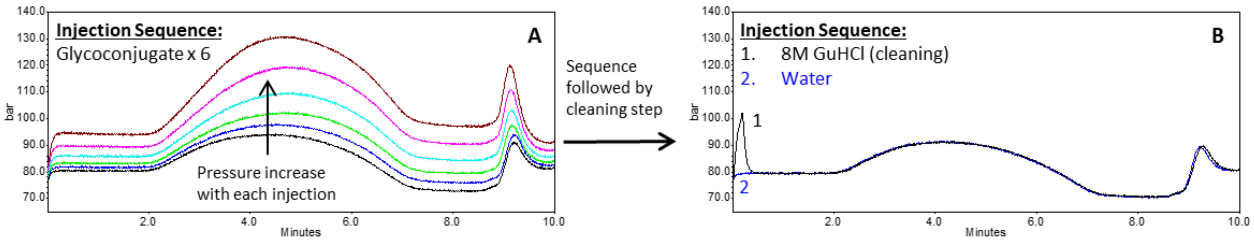

Figure 48. Pressure signal overlay of A) six sequential glycoconjugate sample injections and increasing pressure with each injection and B) an injection of 8 M guanidine hydrochloride and water following glycoconjugate injections showing a return to original operating pressure.

Since the GuHCl cleaning injection is intended for the second dimension RPLC column, the guanidine peak must be transferred from the first dimension via the switching valve in a 2D-LC format. A UV (214 nm) overlay of 8 M GuHCl and water individually injected onto the first dimension SEC is shown in Figure 49. Guanidine can be seen eluting in the inclusion void for this column as expected. Therefore, modification to the timing of the valve switch was required to transfer the guanidine to the second dimension RPLC and clean the column. The valve switch was programmed at 9.5 minutes for all cleaning injections, which required a separate method.

All other 2D-LC parameters remained the same, except for the total run time, which was 30 minutes due to the delayed valve switch.

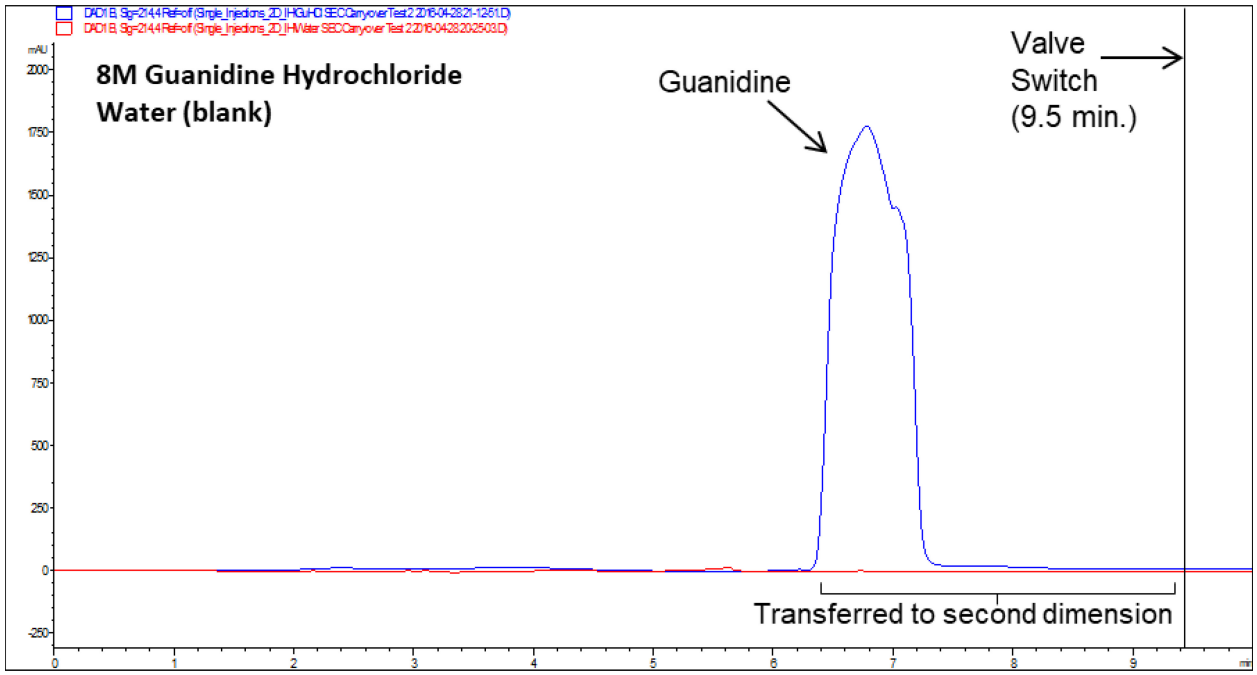

Figure 49 Overlay of first dimension SEC analysis of 8 M guanidine hydrochloride and water showing modified valve switch time to transfer guanidine peak to second dimension for RPLC column cleaning

In addition to the GuHCl flushing injections, a saw-tooth gradient was evaluated to help further elute conjugate. A glycoconjugate spike sample was analyzed by 1D RPLC using the method conditions in Table 13 except for a 1.0 mL/min flow rate and a modified gradient. The gradient program used was based off the final developed conditions from is listed in Table 17, which shortened the main elution program was shortened based off of suggestions in the previous section 3.6.1.

Table 17 Saw-tooth gradient for RPLC analysis of free polysaccharide

Time (m)	Mobile Phase A (%)	Mobile Phase B (%)
0.00	95.0	5.0
1.00	95.0	5.0
6.00	20.0	80.0
8.00	20.0	80.0
8.01	95.0	5.0
9.00	95.0	5.0
10.00	20.0	80.0
11.00	20.0	80.0
11.01	95.0	5.0
12.00	95.0	5.0
13.00	20.0	80.0
14.00	20.0	80.0
14.01	95.0	5.0
15.00	95.0	5.0
16.00	20.0	80.0
17.00	20.0	80.0
17.01	95.0	5.0
20.00	95.0	5.0

Figure 50 shows an overlay of the RPLC analysis of water (blank) and a glycoconjugate spike sample. The asterisk above the peaks in the flushing steps indicating further elution of the glycoconjugate, again indicating strong retention. Though the saw-tooth gradient did not eliminate the increasing pressure issue, it would be implemented to aid in eluting the glycoconjugate from the RPLC column and reduce column build-up.

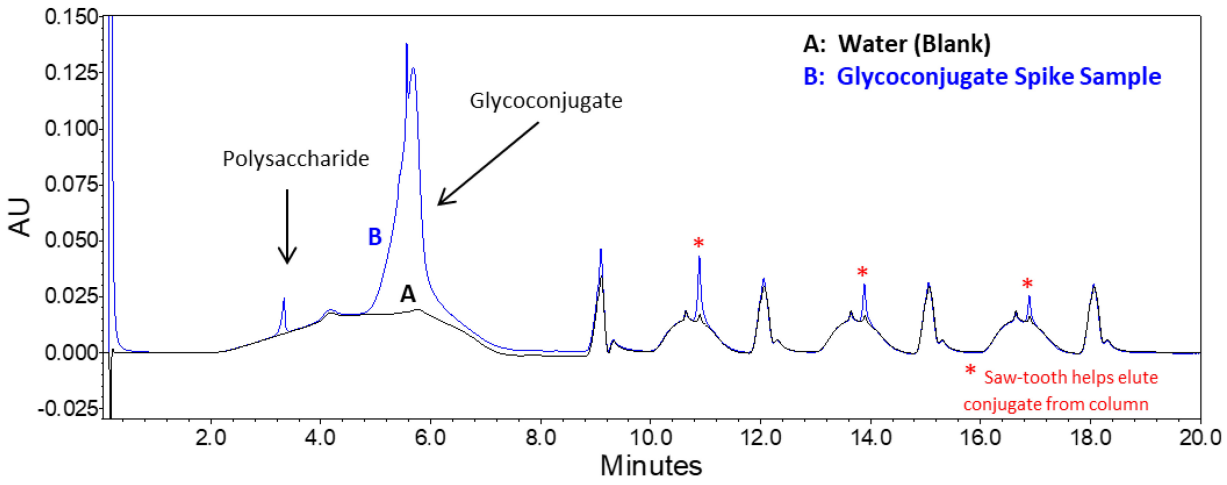

Figure 50. Overlay of UV (214 nm) signal of water (A) and glycoconjugate spike sample (B) using optimized RPLC conditions with a saw-tooth gradient. The implementation of the saw-tooth gradient was shown to help elute the conjugate from the column.

The glycoconjugate insolubility at high organic solvent concentrations coupled with strong retention in the RP-HPLC mode required implementing alternative column flushing conditions using bracketing 8 M guanidine hydrochloride injections. This cleaning step was successful in mitigating the increasing pressure issue due to the inability to elute the conjugate, ensuring a robust method capable of analyzing large sample loads.

Summary

Several challenges were encountered in the final stages of development of the 1D methods for free carrier protein and polysaccharide, and with critical 2D-LC parameters when coupling these methods into a 2D-LC format. The challenges presented highlight how differences in the physical and chemical properties of the individual glycoconjugate components can create analytical challenges during method development. Overcoming these issues prior to performing the method qualification ensured the best possible results. This also provided a better understanding of method robustness under normal operating conditions.

3.7. Qualification of 2D-LC Method

Method conditions for the quantitation of free carrier protein and polysaccharide in glycoconjugate vaccines by 2D-LC (SEC-RPLC) have been established. A method qualification was performed to demonstrate precision, accuracy, linearity, specificity, quantitation limit (QL), and range in accordance with ICH guidelines. Due to instrument limitations, intermediate precision and reproducibility were not evaluated. The purpose of this section is to summarize the experimental design and results from the qualification experiments.

3.7.1. Samples and Preparation

Spiked samples were prepared by adding varying amounts of purified carrier protein and polysaccharide reference standards of known concentrations to CP5-CRM_{197} and CP8-CRM_{197} glycoconjugate samples. This generated spiked samples with a range of free carrier protein and polysaccharide indicated by level "L" and number with increasing amounts. The amount of inherent free carrier protein and polysaccharide were from the established results by CE. These amounts were taken into consideration when targeting levels and calculating theoretical concentrations for accuracy. All standards and samples used in the qualification are listed in Table 18 with their respective spike levels. (Note, values are rounded for display purposes) Free carrier protein ranged from 0.013 to 0.199 mg/mL (1.3 to 19.8%) for CP5- CRM_{197} and 0.008 – 0.190 mg/mL (0.8 – 20.9%) for CP8- CRM_{197}. Free polysaccharide ranged from 0.123 to 0.427 mg/mL (12.8 to 44.4%) for CP5- CRM_{197} and 0.018 – 0.316 mg/mL (1.6 – 27.4%) for CP8- CRM_{197}. The differing ranges of free carrier protein and polysaccharide between the serotypes at the lowest level are due the inherent amount in each from the conjugation process. CP8-CRM_{197} showed lower amounts of free protein and polysaccharide compared to CP5-

CRM_{197}. Also, because the range varies between the serotypes and the performance observed at the lowest level, the lowest level reported will not necessarily be "L1".

Table 18. Spike Sample Information for Method Qualification

Sample ID	Carrier Protein Description			Polysaccharide Description		
	Free mg/mL	Total mg/mL	% Free	Free mg/mL	Total mg/mL	% Free
Carrier Protein Standard	n/a	5.4	n/a	n/a	n/a	n/a
CP5 Polysaccharide Standard	n/a	n/a	n/a	n/a	1.0	n/a
CP8 Polysaccharide Standard	n/a	n/a	n/a	n/a	1.0	n/a
CP5-CRM Spike L1	0.013	1.0	1.3	0.123	1.0	12.8
CP5-CRM Spike L2	0.016	1.0	1.6	0.138	1.0	14.4
CP5-CRM Spike L3	0.033	1.0	3.3	0.161	1.0	16.8
CP5-CRM Spike L4	0.056	1.0	5.7	0.190	1.0	19.9
CP5-CRM Spike L5	0.080	1.0	8.0	0.215	1.0	22.6
CP5-CRM Spike L6	0.113	1.0	11.3	0.265	1.0	27.8
CP5-CRM Spike L7	0.146	1.0	14.8	0.316	0.9	33.2
CP5-CRM Spike L8	0.176	1.0	17.7	0.369	1.0	38.8
CP5-CRM Spike L9	0.199	1.0	19.8	0.427	1.0	44.4
CP8-CRM Spike L1	0.008	1.0	0.8	0.018	1.2	1.6
CP8-CRM Spike L2	0.011	1.0	1.1	0.034	1.2	2.9
CP8-CRM Spike L3	0.028	1.0	2.8	0.052	1.2	4.4
CP8-CRM Spike L4	0.051	1.0	5.1	0.090	1.2	7.7
CP8-CRM Spike L5	0.072	1.0	7.2	0.113	1.2	9.7
CP8-CRM Spike L6	0.109	1.0	11.0	0.162	1.2	14.0
CP8-CRM Spike L7	0.141	1.0	14.2	0.214	1.2	18.4
CP8-CRM Spike L8	0.173	1.0	17.5	0.264	1.2	22.8
CP8-CRM Spike L8	0.206	1.0	20.9	0.316	1.2	27.4

3.7.2. COMPOUNDS AND REAGENTS

The compounds and reagents used are listed in Table 19.

Table 19 Compounds and Reagents

Item	Manufacturer	Catalog Number
Sodium Phosphate Dibasic Heptahydrate, ACS Grade	Fisher Scientific (Hampton, NH)	S373
Sodium phosphate monobasic monohydrate, ACS Grade	Millipore-Sigma (Burlington, MA)	71504
Ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), ACS Grade	Millipore-Sigma (Burlington, MA)	E4884
Sodium Chloride, FCC/USP	Fisher Scientific (Hampton, NH)	S640
Trifluoracetic Acid, ReagentPlus (99%)	Millipore-Sigma (Burlington, MA)	T6508
Acetonitrile (ACN), ACS Grade	Fisher Scientific (Hampton, NH)	AX0145
Purified Water	Milli-Q Water	n/a
Carrier Protein (CRM_{197}) Reference Standard	In-house	n/a
CP5 and CP8 Polysaccharide Reference Standard	In-house	n/a
CP5- CRM_{197} and CP8- CRM_{197} Glycoconjugate	In-house	n/a

3.7.3. EQUIPMENT AND SOFTWARE

The 2D-LC instrument was a demo supplied by Agilent Technologies, Inc. and is commercially available. The Infinity II series demo modules comprised the second dimension and consisted of an 8-port/2-position switching valve (G1170A), 1290 high speed binary pump (G7120A), and diode array detector (G7117B). The demo modules were then coupled to an existing Agilent 1260/1290 HPLC consisting of a 1260 infinity quaternary pump (G5611A), 1290 Infinity (G4226A), and 1260 multi-wavelength detector (G1365D). Agilent's 2D-LC software was utilized to control the instrument, program methods, and data analysis. Data was imported into Waters Empower3 data acquisition system for quantitative analysis and archiving. An Acquity UPLC BEH, 4.6 x 150 mm, 1.7 µm (186005225) from Waters and Poroshell 300SB-C3, 2.1 x 75 mm, 5 µm (660750-909) from Agilent were used for analysis.

3.7.1. METHOD DESCRIPTION

The method was used for the quantitation of free carrier protein and polysaccharide in glycoconjugate vaccines by 2D-LC (SEC-RPLC). A sample is injected onto an SEC column and the carrier protein is separated from the co-eluting glycoconjugate and polysaccharide based on hydrodynamic radius. The response is detected by an ultraviolet (UV) detector. The SEC eluent flows to a switching valve equipped with a sample loop that switched and transfers the co-eluting glycoconjugate and polysaccharide peaks to the second dimension for further analysis. The polysaccharide is then separated from glycoconjugate using an ascending gradient of acetonitrile. The response is detected by a UV detector. 2D-LC method parameters are listed in Table 20.

Table 20: 2D-LC Method Parameters (Analysis)

Parameter	1st Dimension – SEC	2nd Dimension - RP		
Pump	1290 Infinity	1290 Infinity II High Speed Pump		
Autosampler	1290 Infinity			
Detector	1290 Infinity Diode Array	1290 Infinity II Diode Array		
Valve	1290 Infinity Valve Drive (8-port/2-position)			
Column	Waters Acquity UPLC BEH, 4.6 x 150 mm, 1.7 µm (186005225)	Agilent Poroshell 300SB-C3, 2.1 x 75 mm, 5 µm (660750-909)		
Mobile Phase	30 mM Sodium Phosphate, 150 mM Sodium Chloride, pH 6.7	MPA: 0.10% TFA in Water MPB: 0.085% TFA in Acetonitrile		
Flow Rate	0.3 mL/min	1.0 mL/min		
Gradient	Isocratic	Gradient		
		Time, min.	%A	%B
		0.00	95	5
		2.00	95	5
		7.00	25	75
		9.00	25	75
		9.01	95	5
		10.00	95	5
		Saw tooth gradient x3 to 20.5 min		
Detection Mode	UV 280 nm, Peak width = 0.05 min	UV 214 nm, Peak width = 0.013 min		
Column Temp.	30° C	50° C		
Valve Configuration	See Figure 29			
Valve Loop volume	900 µL			
Valve Switch Time	5.5 min.			
Injection Volume	Standard = variable, Glycoconjugate conjugate sample = 5 µL			
Total Runtime	26 min			

A standard curve was generated to quantitate the concentration of free carrier protein and polysaccharide. Purified carrier protein and polysaccharide reference standards of known concentrations were mixed and diluted in purified water to prepare stock standard solutions. These stock standard solutions were directly injected using variable injection volumes from 0.5 to 5.3 µL to achieve a column load ranging from 0.04 to 1.27 µg carrier protein and 0.05 to 2.12 µg polysaccharide. Glycoconjugate samples were prepared by filtering 0.5 mL of sample through a 0.2 µm syringe filter (PALL, 4454). The filtrate was then transferred to an HPLC vial and placed onto the autosampler for analysis. The glycoconjugate sample was injected at 5 µL. Figure 51 shows an example chromatogram of the first and second dimension analysis of the standard mix overlaid with a CP5-CRM_{197} sample.

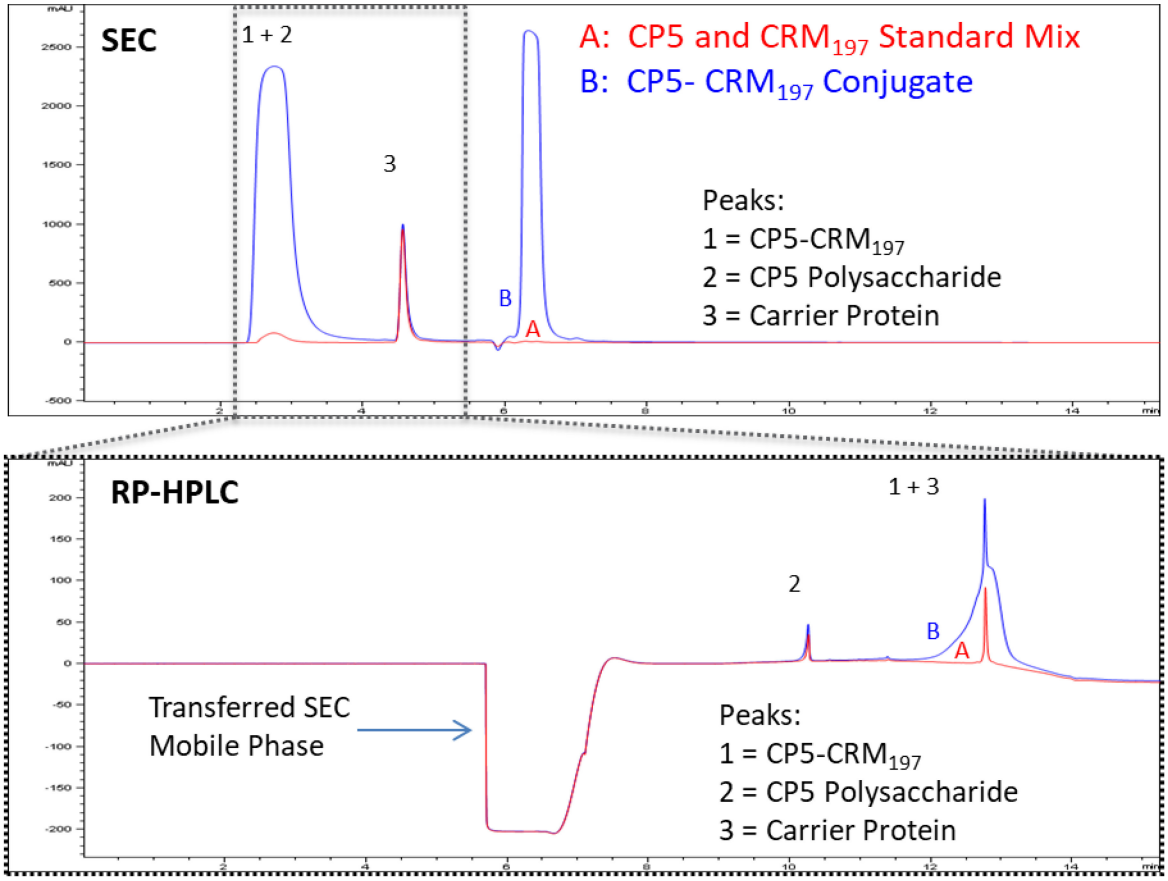

Figure 51. SEC (1st dimension) and RP-HPLC (2nd dimension) chromatogram of a CP5 polysaccharide and CRM_{197} carrier protein mix overlaid with a CP5-CRM_{197} spike sample. Dotted region transferred to RP-HPLC for separation and quantitation of free polysaccharide.

A cleaning injection with 8 M guanidine hydrochloride (GuHCl) is performed every five samples to remove any retained glycoconjugate from the second dimension HPLC column. GuHCl is injected neat at 20 µL. As the elution time of GuHCl is different than the glycoconjugate and polysaccharide, modified parameters are needed to transfer the GuHCl to the second dimension. The method parameters for column cleaning are listed in Table 21.

Table 21: 2D-LC Method Parameters (Cleaning)

Parameter	1st Dimension – SEC	2nd Dimension - RP
Valve Switch Time	9.5 min.	
Total Runtime	30 min	

To calculate free carrier protein, the peak is integrated in the chromatogram. A linear regression analysis is then performed using the column load of each standard (x) and the area count (y) of

the standard to produce a line equation, calculating the intercept (c), slope (m), and correlation coefficient (R). The column load (x) for a sample is then calculated using the area count (y) in the sample and the line fit equation. The column load is then divided by the injection volume (µL) to calculate concentration of free carrier protein. To calculate the final free carrier protein, the concentration of carrier protein is divided by the total protein concentration (obtained by the Modified-Lowry method). This ratio is multiplied by 100 and expressed as a percentage of free carrier protein. The same steps are applied for the calculation of free polysaccharide. The exceptions are that the second dimension peak areas of the polysaccharide are used, and the concentration of the total polysaccharide (obtained by PAHBAH) is used for the calculation of percent free polysaccharide. For the purposes of method performance evaluation, only the mg/mL concentration for free carrier protein and polysaccharide will be used.

3.7.2. Assessment of Method Performance

The experimental design and results for each qualification characteristic are discussed in detail in the following sections.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. For this qualification, Specificity was evaluated by demonstrating that the sample matrix contains no measurable peaks in the region of interest. Figure 52 shows an overlay from the 2D analysis of a glycoconjugate spike sample and placebo.

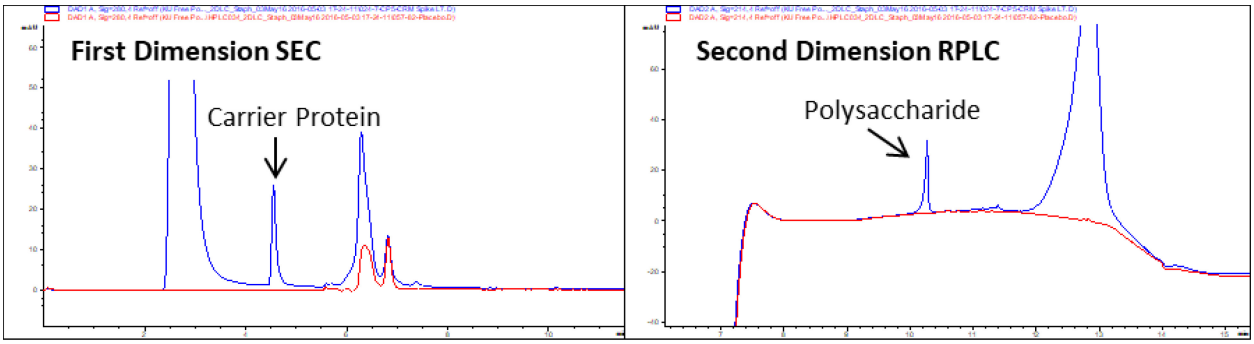

Figure 52 2D-LC analysis of placebo (blank) and glycoconjugate spike sample showing no interfering peaks in the region of the carrier protein or polysaccharide peaks.

No interference or contribution from the placebo was observed in either dimension.

Linearity (Standard)

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. As purified reference standards of carrier protein and polysaccharides are used to generate a standard curve for quantitation in glycoconjugate samples, linearity of each component was evaluated for both standards and glycoconjugate samples. This section describes the evaluation of standards while the following section, Linearity (Sample), will describe the linearity evaluation in glycoconjugate samples.

Carrier protein and polysaccharide reference standards were prepared by mixing and diluting in water. Separate mixtures were prepared for both serotypes qualified. The injection volume was varied to achieve a target column load. The first dimension column load for carrier protein ranged from 0.04 to 1.01 µg. The second dimension range column load range from 0.05 to 1.68 µg for the CP5 polysaccharide standard and 0.16 to 1.68 µg for the CP8 polysaccharide standard. One injection was made for each level.

The linearity of the carrier protein standard in the first dimension was evaluated. Note, data and results for the carrier protein appeared similar whether mixed with CP5 or CP8 polysaccharide serotypes. Therefore, only data and results from the mixture with CP5 polysaccharide standard are shown for simplicity. Linearity data for carrier protein from the first dimension are shown in Table 22. To evaluate linearity of the carrier protein standard, a linearity plot was constructed using the peak area (y-axis) and column load in µg (x-axis) as shown in Figure 53. The plot was inspected, which appeared linear. A linear least squares regression was then performed with residual plots. The output of the regression analysis is listed in Table 23 and the residual plot shown in Figure 54 was inspected for patterns.

Table 22 Linearity data for Carrier Protein Standard from the first dimension SEC

Sample ID	Injection Volume (µL)	Column Load – Carrier Protein (µg)	Area (280 nm)
CP5 Standard Mix L1	0.5	0.04	24864
CP5 Standard Mix L2	0.6	0.05	30282
CP5 Standard Mix L3	1.6	0.13	82294
CP5 Standard Mix L4	3.1	0.25	166161
CP5 Standard Mix L5	4.4	0.35	240715
CP5 Standard Mix L6	2.1	0.50	364919
CP5 Standard Mix L7	2.7	0.65	476231
CP5 Standard Mix L8	3.3	0.80	594748
CP5 Standard Mix L9	4.2	1.01	770285

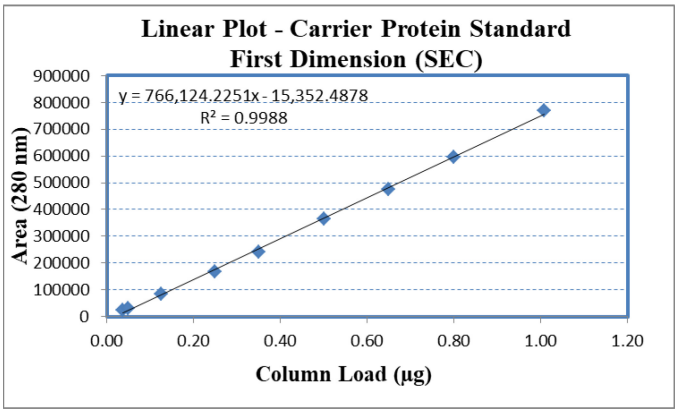

Figure 53 Linear plot of the carrier protein standard from the first dimension SEC

Table 23 Regression data for carrier protein standard from the first dimension SEC

Correlation Coefficient (R)	0.9994

Slope	766283
Intercept	-15462
Residual sum of Squares	6.62E+08

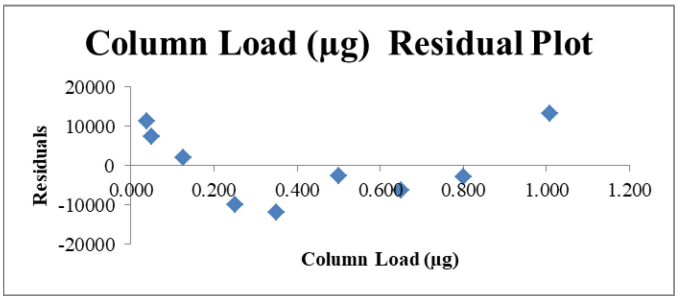

Figure 54 Residual plot of the carrier protein standard from the first dimension SEC

The linearity of the CP5 and CP8 polysaccharide standards from the second dimension was then evaluated. Linearity data are shown in Table 24 for the CP5 polysaccharide standard and Table 26 for CP8 polysaccharide. To evaluate linearity of the polysaccharide standards, linearity plots were constructed using the peak area (y-axis) and column load in µg (x-axis) as shown in Figure 55 for CP5 and in Figure 57 for CP8. The plots were inspected, which appeared linear. A linear least squares regression was then performed with residual plots. The output of the regression analysis is listed in Table 25 for CP5 and in Table 27 for CP8. The residual plots were inspected for patterns and are shown in Figure 56 for CP5 and Figure 58 for CP8.

Table 24 Linearity data for CP5 Polysaccharide Standard from the second dimension RPLC

Sample ID	Injection Volume (µL)	Column Load – CP5 Polysaccharide (µg)	Area (214 nm)
CP5 Standard Mix L1	0.5	0.05	5297
CP5 Standard Mix L2	0.6	0.06	6105
CP5 Standard Mix L3	1.6	0.16	16790
CP5 Standard Mix L4	3.1	0.31	32575
CP5 Standard Mix L5	4.4	0.44	47133
CP5 Standard Mix L6	2.1	0.84	85966
CP5 Standard Mix L7	2.7	1.08	111159
CP5 Standard Mix L8	3.3	1.32	136074
CP5 Standard Mix L9	4.2	1.68	172956

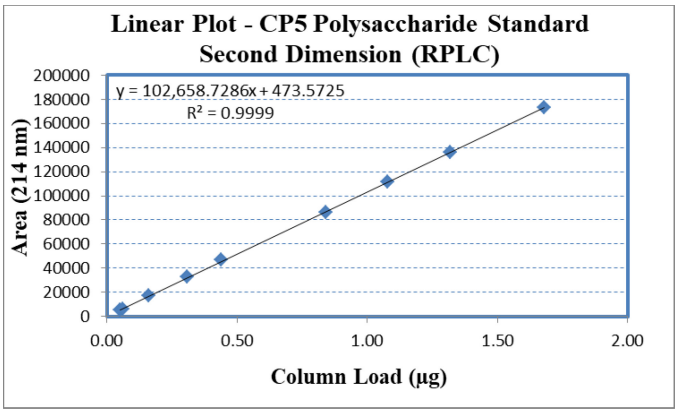

Figure 55 Linear plot of the CP5 polysaccharide standard from the second dimension RPLC

Table 25: Regression Data for CP5 Polysaccharide Standard from the Second Dimension RPLC

Correlation Coefficient (R)	0.9999
Slope	102659
Intercept	474
Residual sum of Squares	3.27E+06

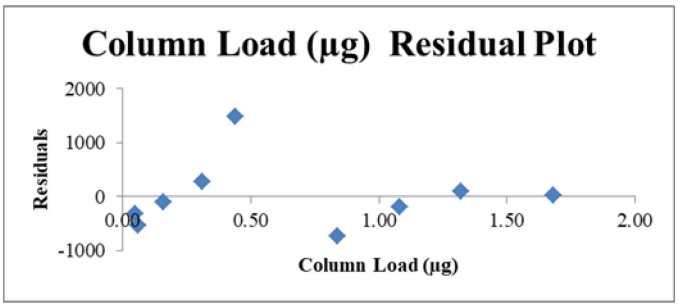

Figure 56 Residual plot of the CP5 polysaccharide standard from the second dimension RPLC

Table 26 Linearity data for CP8 Polysaccharide Standard from the second dimension RPLC

Sample ID	Injection Volume (µL)	Column Load – CP8 Polysaccharide (µg)	Area (214 nm)
CP8 Standard Mix L1	1.6	0.16	14331
CP8 Standard Mix L2	3.1	0.31	31100
CP8 Standard Mix L3	4.4	0.44	47488
CP8 Standard Mix L4	2.1	0.84	87069
CP8 Standard Mix L5	2.7	1.08	114320
CP8 Standard Mix L6	3.3	1.32	138432
CP8 Standard Mix L7	4.2	1.68	175735

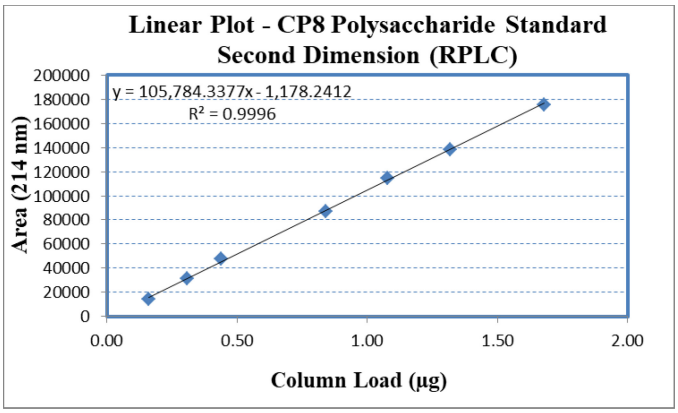

Figure 57 Linear plot of the CP8 polysaccharide standard from the second dimension RPLC

Table 27 Regression Data for CP8 Polysaccharide Standard from the Second Dimension RPLC

Correlation Coefficient	0.9998
Slope	105784
Intercept	-1178
Residual sum of Squares	9.36E+06

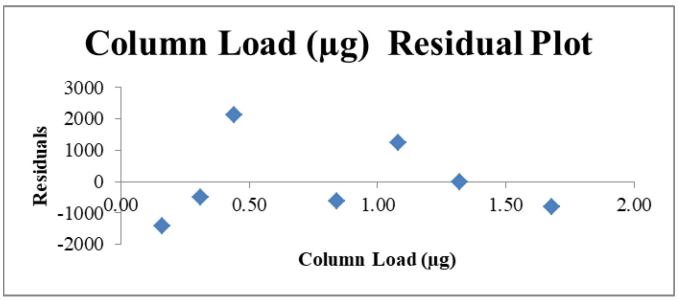

Figure 58 Residual plot of the CP8 Polysaccharide standard from the second dimension RPLC

Linearity was demonstrated for the carrier protein in the first dimension from 0.04 to 1.01 µg. Linearity was demonstrated in the second dimension for the CP5 polysaccharide standard from 0.05 to 1.68 µg and for the CP8 polysaccharide from 0.16 to 1.68 µg.

Linearity (Sample)

Linearity evaluation of the reference standards were described in the previous section, Linearity (Standard). This section describes the linearity evaluation of the free carrier protein and polysaccharide in CP5-CRM_{197} and CP8-CRM_{197} glycoconjugate samples. The first dimension column load for carrier protein ranged from 0.28 to 0.99 µg for CP5-CRM_{197} and 0.14 to 1.03 µg

for CP8-CRM_{197}. The second dimension column load for the respective polysaccharide ranged from 0.61 to 2.14 µg for the CP5-CRM_{197} and 0.17 to 1.58 µg for the CP8-CRM_{197}. Samples were injected at 5 µL and one injection was made for each level.

The linearity of the free carrier protein in CP5-CRM_{197} and CP8-CRM_{197} from the first dimension was evaluated. Linearity data are shown in Table 28 for CP5-CRM_{197} and Table 30 for CP8-CRM_{197}. To evaluate linearity of the glycoconjugate samples, linearity plots were constructed using the peak area (y-axis) and column load in µg (x-axis) as shown in Figure 59 for CP5-CRM_{197} and in Figure 61 or CP8-CRM_{197}. The plots were inspected, which appeared linear. A linear least squares regression was then performed with residual plots. The output of the regression analysis is listed in Table 29 for CP5-CRM_{197} and in Table 31 for CP8-CRM_{197}. The residual plots were inspected for patterns and are shown in Figure 60 for CP5-CRM_{197} and Figure 62 for CP8-CRM_{197}.

Table 28 Linearity data for Carrier Protein in CP5-CRM_{197} from the first dimension SEC

Sample ID	Injection Volume (µL)	Column Load – Carrier Protein in CP5-CRM_{197} (µg)	Area (280 nm)	Carrier Protein in CP5-CRM_{197} (mg/mL)
CP5-CRM Spike L4	5	0.28	169052	0.056
CP5-CRM Spike L5	5	0.40	257458	0.080
CP5-CRM Spike L6	5	0.56	382607	0.113
CP5-CRM Spike L7	5	0.73	514064	0.146
CP5-CRM Spike L8	5	0.88	633078	0.176
CP5-CRM Spike L9	5	0.99	665639	0.199

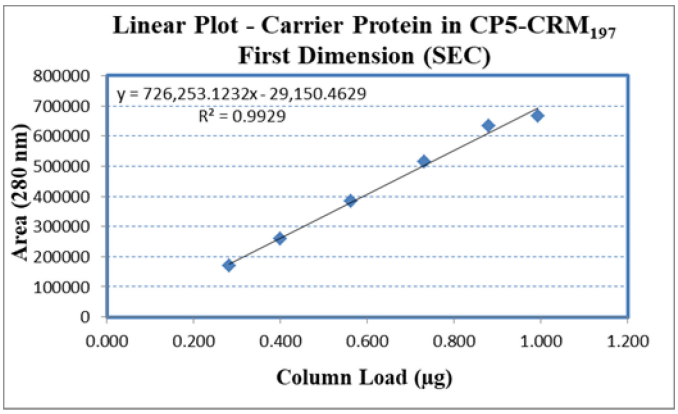

Figure 59 Linear plot of the carrier protein in CP5-CRM$_{197}$ from the first dimension SEC

Table 29 Regression data for carrier protein in CP5-CRM$_{197}$ from the first dimension

Correlation Coefficient	0.9964
Slope	726253
Intercept	-29150
Residual sum of Squares	1.46E+09

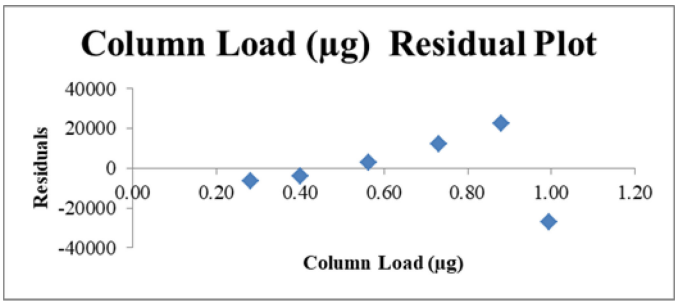

Figure 60 Residual plot of the carrier protein in CP5-CRM$_{197}$ from the first dimension SEC

Table 30 Linearity data for carrier protein in CP8-CRM$_{197}$ from the first dimension SEC

Sample ID	Injection Volume (µL)	Theoretical Column Load (µg)	Area (280 nm)	Carrier Protein in CP8-CRM$_{197}$ (mg/mL)
CP8-CRM Spike L3	5	0.14	95654	0.028
CP8-CRM Spike L4	5	0.25	174832	0.051
CP8-CRM Spike L5	5	0.36	254384	0.072
CP8-CRM Spike L6	5	0.71	516117	0.141
CP8-CRM Spike L7	5	0.86	647480	0.173
CP8-CRM Spike L8	5	1.03	769130	0.206

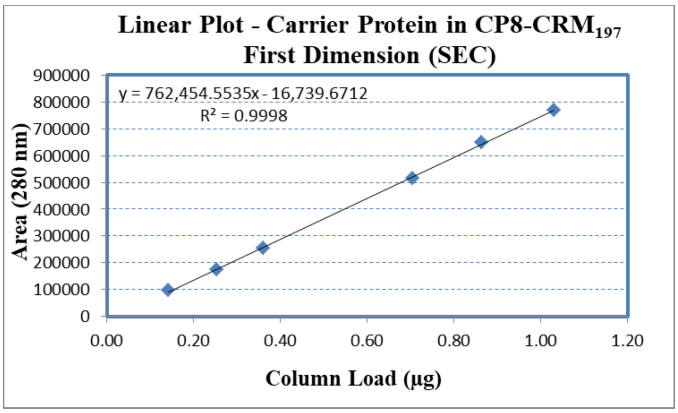

Figure 61 Linear plot of the carrier protein in CP8-CRM$_{197}$ from the first dimension SEC

Table 31 Regression data for carrier protein in CP8-CRM$_{197}$ from the first dimension SEC

Correlation Coefficient	0.9998
Slope	762455
Intercept	-16740
Residual sum of Squares	7.98E+07

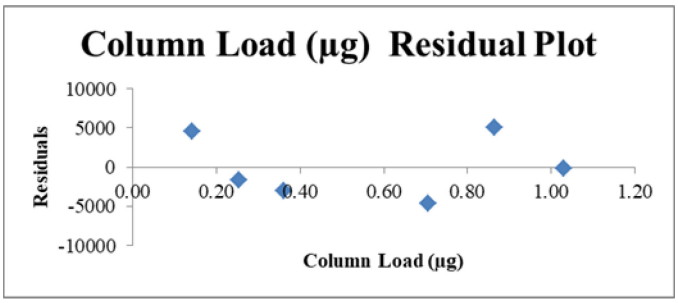

Figure 62 Residual plot of the carrier protein in CP8-CRM$_{197}$ from the first dimension SEC

The linearity of the respective free polysaccharide in CP5-CRM$_{197}$ and CP8-CRM$_{197}$ from the second dimension was then evaluated. Linearity data are shown in Table 32 for CP5-CRM$_{197}$ and Table 34 for CP8-CRM$_{197}$. To evaluate linearity of the glycoconjugate samples, linearity plots were constructed using the peak area (y-axis) and column load in µg (x-axis) as shown in Figure 63 for CP5-CRM$_{197}$ and Figure 65 for CP8-CRM$_{197}$. The plots were inspected, which appeared linear. A linear least squares regression was then performed with residual plots. The output of the regression analysis is listed in Table 33 for CP5-CRM$_{197}$ and in Table 35 for

CP8-CRM$_{197}$. The residual plots were inspected for patterns and are shown in Figure 64 for CP5-CRM$_{197}$ and Figure 66 for CP8-CRM$_{197}$.

Table 32 Linearity data for polysaccharide in CP5-CRM$_{197}$ from the second dimension RPLC

Sample ID	Injection Volume (µL)	Column Load – Polysaccharide in CP5-CRM$_{197}$ (µg)	Area (214 nm)	Polysaccharide in CP5-CRM$_{197}$ (mg/mL)
CP5-CRM Spike L1	5	0.61	67788	0.123
CP5-CRM Spike L2	5	0.69	73383	0.138
CP5-CRM Spike L3	5	0.80	86849	0.161
CP5-CRM Spike L4	5	0.95	108096	0.190
CP5-CRM Spike L5	5	1.08	121225	0.215
CP5-CRM Spike L6	5	1.32	153492	0.265
CP5-CRM Spike L7	5	1.58	184761	0.316
CP5-CRM Spike L8	5	1.85	219769	0.369
CP5-CRM Spike L9	5	2.14	249023	0.427

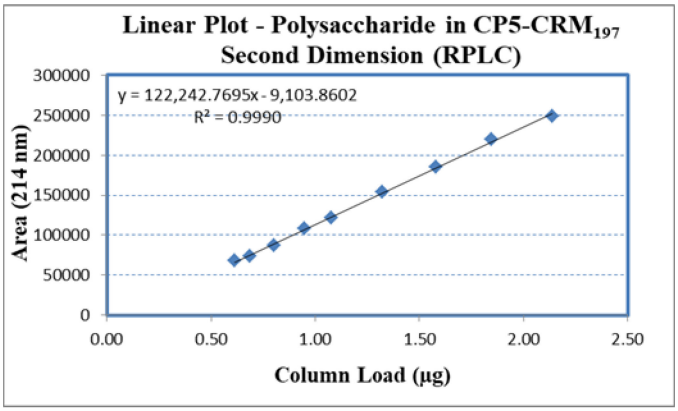

Figure 63 Linear plot of the polysaccharide in CP5-CRM$_{197}$ from the second dimension RPLC

Table 33 Regression Data for polysaccharide in CP5-CRM$_{197}$ from the second dimension RPLC

Correlation Coefficient	0.9995
Slope	122243
Intercept	-9104
Residual sum of Squares	3.54E+07

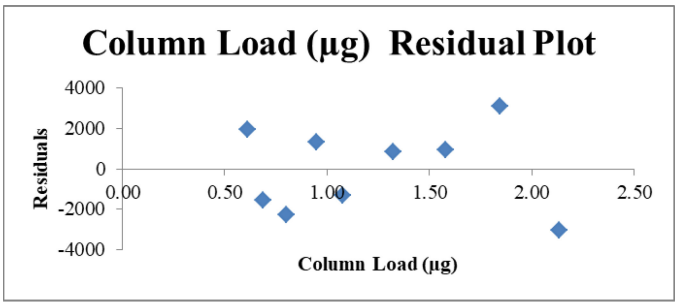

Figure 64 Residual plot of the polysaccharide in CP5-CRM$_{197}$ from the second dimension RPLC

Table 34 Linearity data for polysaccharide in CP8-CRM$_{197}$ from the second dimension RPLC

Sample ID	Injection Volume (µL)	Column Load – Polysaccharide in CP8-CRM$_{197}$ (µg)	Area (214 nm)	Polysaccharide in CP8-CRM$_{197}$ (mg/mL)
CP8-CRM Spike L2	5	0.17	17059	0.034
CP8-CRM Spike L3	5	0.26	26230	0.052
CP8-CRM Spike L4	5	0.45	47614	0.090
CP8-CRM Spike L5	5	0.57	63393	0.113
CP8-CRM Spike L6	5	0.81	89158	0.162
CP8-CRM Spike L7	5	1.07	124575	0.214
CP8-CRM Spike L8	5	1.32	152922	0.264
CP8-CRM Spike L9	5	1.58	185459	0.316

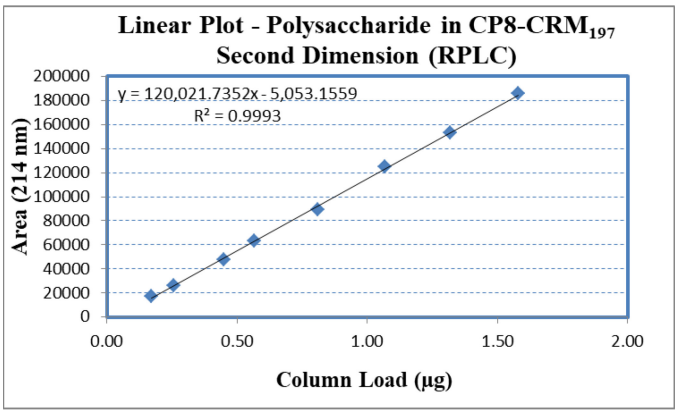

Figure 65 Linear plot of the polysaccharide in CP8-CRM$_{197}$ from the second dimension RPLC

Table 35 Regression data for polysaccharide in CP8-CRM$_{197}$ from the second dimension RPLC

Correlation Coefficient	0.9997
Slope	120022
Intercept	-5053
Residual sum of Squares	1.77E+07

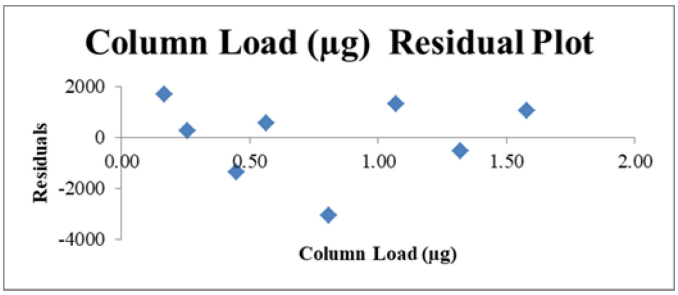

Figure 66 Residual plot of the carrier protein in CP8-CRM$_{197}$ from the second dimension RPLC

Linearity was demonstrated for the free carrier protein in the first dimension from 0.28 to 0.99 µg for CP5-CRM$_{197}$ and 0.14 to 1.03 µg for CP8-CRM$_{197}$. Using a constant injection volume of 5 µL, this equated to a free carrier protein concentration range of 0.056 to 0.199 mg/mL for CP5-CRM$_{197}$ and 0.028 to 0.206 mg/mL for CP8-CRM$_{197}$.

Linearity was demonstrated in the second dimension for the respective free polysaccharide from 0.61 to 2.14 µg for the CP5-CRM$_{197}$ and 0.17 to 1.58 µg for the CP8-CRM$_{197}$. Using a constant injection volume of 5 µL, this equated to a free polysaccharide concentration range of 0.123 to 0.427 mg/mL for CP5-CRM$_{197}$ and 0.034 to 0.316 mg/mL for CP8-CRM$_{197}$. The significant difference in lower limit for linearity in polysaccharide between the serotypes is due to the differing amounts of free polysaccharide inherently present in the sample.

Precision

Method precision is a measure of the degree of repeatability of the method for single reportable measurements from multiple sample preparations under normal operating conditions. For this qualification, method precision was performed by testing three independent preparations (P1-P3) of samples containing three levels of free carrier protein in CP5-CRM$_{197}$ and CP8-CRM$_{197}$ (low, mid, and high). These samples also contained three levels of the respective free polysaccharide

(low, mid, and high). This allowed for carrier protein and polysaccharide to be evaluated in the same injection. Each replicate was injected once for a total of 9 determinations.

The free carrier protein concentrations from the analysis of CP5-CRM_{197} and CP8-CRM_{197} are tabulated in Table 36 and Table 38, respectively. The individual results (mg/mL) were normalized by dividing the reported result against the average result for that level and are tabulated in Table 37 for CP5-CRM_{197} and Table 39 for CP8-CRM_{197}. The overall mean, standard deviation, and percent relative standard deviation (%RSD) were calculated for all results. The 95% Confidence Interval (CI) was only calculated for the results that were not normalized. The overall %RSD for free carrier protein (mg/mL) is reported as the method precision.

Table 36 Assessment of method precision for carrier protein in CP5-CRM_{197}

Sample ID	P1 (mg/mL)	P2 (mg/mL)	P3 (mg/mL)	Mean (mg/mL)	Standard Deviation	% RSD	95% Confidence Interval (±)
CP5-CRM Spike L4	0.051	0.051	0.051	0.051	0.000	0.4	0.000
CP5-CRM Spike L6	0.101	0.101	0.097	0.100	0.002	2.2	0.005
CP5-CRM Spike L9	0.179	0.179	0.181	0.180	0.001	0.8	0.003

Table 37: Assessment of method precision (normalized) for carrier protein in CP5-CRM_{197}

Sample ID	P1	P2	P3
CP5-CRM Spike L4	1.00	1.00	1.00
CP5-CRM Spike L6	1.01	1.01	0.97
CP5-CRM Spike L9	1.00	1.00	1.01
Mean (n=9)			1.00
Standard Deviation (n=9)			0.01
Overall %RSD (n=9)			1.2

Table 38 Assessment of method precision for carrier protein in CP8-CRM_{197}

Sample ID	P1 (mg/mL)	P2 (mg/mL)	P3 (mg/mL)	Mean (mg/mL)	Standard Deviation	% RSD	95% Confidence Interval (±)
CP8-CRM Spike L3	0.024	0.024	0.024	0.024	0.000	0.1	0.000
CP8-CRM Spike L6	0.090	0.091	0.091	0.091	0.001	0.7	0.002
CP8-CRM Spike L9	0.180	0.177	0.178	0.178	0.002	1.0	0.004

Table 39 Assessment of method precision (normalized) for carrier protein in CP8-CRM$_{197}$

Sample ID	P1	P2	P3
CP8-CRM Spike L3	1.00	1.00	1.00
CP8-CRM Spike L6	0.99	1.00	1.01
CP8-CRM Spike L9	1.01	0.99	1.00
Mean (n=9)			1.00
Standard Deviation (n=9)			0.01
Overall %RSD (n=9)			0.6

The free polysaccharide concentrations from the analysis of CP5-CRM$_{197}$ and CP8-CRM$_{197}$ are tabulated in Table 40 and Table 42, respectively. The individual results (mg/mL) were normalized by dividing the reported result against the average result for that level and are tabulated in Table 41 for CP5-CRM$_{197}$ and Table 43 for CP8-CRM$_{197}$. The overall mean, standard deviation, and percent relative standard deviation (%RSD) were calculated for all results. The 95% Confidence Interval (CI) was only calculated for the results that were not normalized. The overall %RSD for free carrier protein (mg/mL) is reported as the method precision.

Table 40 Assessment of method precision for polysaccharide in CP5-CRM$_{197}$

Sample ID	P1 (mg/mL)	P2 (mg/mL)	P3 (mg/mL)	Mean (mg/mL)	Standard Deviation	% RSD	95% Confidence Interval (±)
CP5-CRM Spike L4	0.116	0.117	0.115	0.116	0.001	0.9	0.002
CP5-CRM Spike L6	0.295	0.293	0.298	0.296	0.002	0.8	0.006
CP5-CRM Spike L9	0.479	0.482	0.478	0.480	0.002	0.4	0.005

Table 41 Assessment of method precision (normalized) for polysaccharide in CP5-CRM$_{197}$

Sample ID	P1	P2	P3
CP5-CRM Spike L4	1.00	1.01	0.99
CP5-CRM Spike L6	1.00	0.99	1.01
CP5-CRM Spike L9	1.00	1.00	1.00
Mean (n=9)			1.00
Standard Deviation (n=9)			0.01
Overall %RSD (n=9)			0.6

Table 42 Assessment of method precision for polysaccharide in CP8-CRM$_{197}$

Sample ID	P1 (mg/mL)	P2 (mg/mL)	P3 (mg/mL)	Mean (mg/mL)	Standard Deviation	% RSD	95% Confidence Interval (±)
CP8-CRM Spike L2	0.033	0.034	0.034	0.034	0.000	1.4	0.001
CP8-CRM Spike L5	0.125	0.126	0.125	0.125	0.001	0.6	0.002
CP8-CRM Spike L9	0.368	0.368	0.365	0.367	0.002	0.5	0.004

Table 43 Assessment of method precision (normalized) for polysaccharide in CP8-CRM$_{197}$

Sample ID	P1	P2	P3
CP8-CRM Spike L2	0.99	1.00	1.01
CP8-CRM Spike L5	1.00	1.01	1.00
CP8-CRM Spike L9	1.00	1.00	0.99
Mean (n=9)			1.00
Standard Deviation (n=9)			0.01
Overall %RSD (n=9)			0.8

Method precision for free carrier protein was demonstrated to be 1.2% for CP5-CRM$_{197}$ and 0.6% for CP8-CRM$_{197}$. Method precision for free polysaccharide was demonstrated to be 0.6% for CP5-CRM$_{197}$ and 0.8% for CP8-CRM$_{197}$.

Accuracy

Accuracy is the closeness of agreement between the acceptable value and the experimentally determined value. For this qualification, accuracy was evaluated by testing three independent preparations (P1-P3) of samples containing three levels of free carrier protein in CP5-CRM$_{197}$ and CP8-CRM$_{197}$ (low, mid, and high). These samples also contained three levels of the respective free polysaccharide (low, mid, and high). Each replicate was injected once for a total of 9 determinations.

The free carrier protein concentrations from the analysis of CP5-CRM$_{197}$ and CP8-CRM$_{197}$ are tabulated in Table 44 and Table 45, respectively. For each sample, the percent recovery (accuracy) was calculated by dividing the measured carrier protein concentration (mg/mL) by the corresponding concentration (mg/mL) and multiplying by 100%. The mean percent recovery is reported as the accuracy.

Table 44 Accuracy assessment of free carrier protein in CP5-CRM$_{197}$

Sample ID	Measured Carrier Protein in CP5-CRM$_{197}$ (mg/mL)	Theoretical Carrier Protein in CP5-CRM$_{197}$ (mg/mL)	%Recovery	Mean %Recovery
CP5-CRM Spike L4 P1	0.051	0.056	90.3	91.8
CP5-CRM Spike L4 P2	0.051	0.056	90.6	
CP5-CRM Spike L4 P3	0.051	0.056	90.9	
CP5-CRM Spike L6 P1	0.101	0.109	92.7	
CP8-CRM Spike L6 P2	0.101	0.109	92.7	
CP5-CRM Spike L6 P3	0.097	0.109	89.3	
CP5-CRM Spike L9 P1	0.179	0.193	92.9	
CP5-CRM Spike L9 P2	0.178	0.193	92.7	
CP5-CRM Spike L9 P3	0.181	0.193	94.1	

Table 45 Accuracy assessment of free carrier protein in CP8-CRM$_{197}$

Sample ID	Measured Carrier Protein in CP8-CRM$_{197}$ (mg/mL)	Theoretical Carrier Protein in CP8-CRM$_{197}$ (mg/mL)	%Recovery	Mean %Recovery
CP8-CRM Spike L3 P1	0.024	0.025	94.6	94.3
CP8-CRM Spike L3 P2	0.024	0.025	94.8	
CP8-CRM Spike L3 P3	0.024	0.025	94.8	
CP8-CRM Spike L6 P1	0.090	0.097	92.4	
CP8-CRM Spike L6 P2	0.091	0.097	93.4	
CP8-CRM Spike L6 P3	0.091	0.097	93.6	
CP8-CRM Spike L9 P1	0.180	0.188	96.0	
CP8-CRM Spike L9 P2	0.177	0.188	94.2	
CP8-CRM Spike L9 P3	0.178	0.188	95.1	

The free polysaccharide concentrations from the analysis of CP5-CRM$_{197}$ and CP8-CRM$_{197}$ are tabulated in Table 46 and Table 47, respectively. For each sample, the percent recovery (accuracy) was calculated by dividing the measured polysaccharide concentration (mg/mL) by the corresponding theoretical concentration (mg/mL) and multiplying by 100%. The mean percent recovery is reported as the accuracy.

Table 46 Accuracy assessment of free polysaccharide in CP5-CRM$_{197}$

Sample ID	Measured Polysaccharide in CP5-CRM$_{197}$ (mg/mL)	Theoretical Polysaccharide in CP5-CRM$_{197}$ (mg/mL)	%Recovery	Mean %Recovery
CP5-CRM Spike L4 P1	0.116	0.113	102.6	109.8

CP5-CRM Spike L4 P2	0.117	0.113	103.7	
CP5-CRM Spike L4 P3	0.115	0.113	101.9	
CP5-CRM Spike L6 P1	0.295	0.265	111.6	
CP8-CRM Spike L6 P2	0.293	0.265	110.9	
CP5-CRM Spike L6 P3	0.298	0.265	112.7	
CP5-CRM Spike L9 P1	0.479	0.418	114.7	
CP5-CRM Spike L9 P2	0.482	0.418	115.4	
CP5-CRM Spike L9 P3	0.478	0.418	114.5	

Table 47 Accuracy assessment of free polysaccharide in CP8-CRM$_{197}$

Sample ID	**Measured Polysaccharide in CP8-CRM$_{197}$ (mg/mL)**	**Theoretical Polysaccharide in CP8-CRM$_{197}$ (mg/mL)**	**%Recovery**	**Mean %Recovery**
CP8-CRM Spike L2 P1	0.033	0.034	97.5	
CP8-CRM Spike L2 P2	0.034	0.034	99.2	
CP8-CRM Spike L2 P3	0.034	0.034	100.2	
CP8-CRM Spike L5 P1	0.125	0.111	112.5	
CP8-CRM Spike L5 P2	0.126	0.111	113.5	109.5
CP8-CRM Spike L5 P3	0.125	0.111	112.4	
CP8-CRM Spike L9 P1	0.368	0.315	117.0	
CP8-CRM Spike L9 P2	0.368	0.315	117.0	
CP8-CRM Spike L9 P3	0.365	0.315	116.0	

Accuracy for free carrier protein was demonstrated to be 91.8% for CP5-CRM$_{197}$ and 91.3% for CP8-CRM$_{197}$. Accuracy for free polysaccharide was demonstrated to be 109.8% for CP5-CRM$_{197}$ and 109.5% for CP8-CRM$_{197}$.

Detection Limit

The detection limit can be determined by comparing measured signal from samples with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio of 3 is generally acceptable. As the lowest level of both standards and samples evaluated were all significantly above a signal-to-noise level of 3, the detection limit was not established.

Quantitation Limit

The quantitation limit in a method exhibiting baseline noise can be determined by comparing measured signals from samples with those of blank samples and establishing the minimum concentration at which the analyte can be reliable quantified. A typical signal-to-noise ratio for the quantitation limit is 10:1. The USP signal-to-noise ratio was calculated using Empower software for the carrier protein and polysaccharide peaks in the low-level spike sample. These results are summarized in Table 48 and Table 49.

Table 48 Carrier Protein Peak Signal-to-noise in Low Level CP5-CRM$_{197}$ and CP8-CRM$_{197}$

Sample ID	USP Signal-to-Noise	Column Load – Carrier Protein (µg)	Area (280 nm)	Carrier Protein (mg/mL)
CP5-CRM Spike L4	1309	0.28	169052	0.056
CP8-CRM Spike L3	1316	0.14	95654	0.028

Table 49 Polysaccharide Signal-to-noise Peak in Low Level CP5-CRM$_{197}$ and CP8-CRM$_{197}$

Sample ID	USP Signal-to-Noise	Column Load – Polysaccharide (µg)	Area (214 nm)	Polysaccharide (mg/mL)
CP5-CRM Spike L1	346	0.61	67788	0.123
CP8-CRM Spike L2	280	0.17	17059	0.034

The signal-to-noise ratios were all significantly greater than the typical 10:1 limit and did not appear to be the limiting parameter for the evaluation of the QL. Alternatively, the QL of an individual analytical procedure is the minimum level at which the analyte can be quantified with acceptable accuracy and precision. Therefore, the QL was established to be the lowest levels evaluated for accuracy and precision, which were acceptable for a residual quantitation method.

The QL for free carrier protein was demonstrated to be 0.056 mg/mL for CP5-CRM$_{197}$ and 0.028 mg/mL for CP8-CRM$_{197}$. The QL for free polysaccharide was demonstrated to be 0.123 mg/mL for CP5-CRM$_{197}$ and 0.034 mg/mL for CP8-CRM$_{197}$.

Range

Range is the region that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure. The range for free carrier protein was demonstrated from 0.056 to 0.199 mg/mL (5.7 – 19.8%) for CP5-CRM_{197} and 0.028 to 0.206 mg/mL (2.8 – 20.9%) for CP8-CRM_{197}. The range for free polysaccharide was demonstrated from 0.123 to 0.427 mg/mL (12.8 – 44.4%) for CP5-CRM_{197} and 0.034 to 0.316 mg/mL (2.9 – 27.4%) for CP8-CRM_{197}.

Summary

The method was qualified in accordance with ICH guidelines demonstrating precision, accuracy, linearity, specificity, quantitation limit (QL), and range. Results from the qualification results are summarized in Table 50.

Table 50 Summary of Qualification Results for CP5-CRM$_{197}$ and CP8-CRM$_{197}$

Parameter	Free Carrier Protein		Free Polysaccharide	
	CP5-CRM$_{197}$	**CP8-CRM$_{197}$**	**CP5-CRM$_{197}$**	**CP8-CRM$_{197}$**
Linearity Standard	0.04 – 1.01 µg (load) R = 0.9994 Slope = 766283 Intercept = -15462		0.05 – 1.68 µg (load) R = 0.9999 Slope = 102659 Intercept = 474	0.16 – 0.1.68 µg (load) R = 0.99948 Slope = 105784 Intercept = -1178
Linearity Sample	0.28 – 0.99 µg (load) 0.056 – 0.199 mg/mL R = 0.9964 Slope = 726253 Intercept = -29150	0.14 – 1.03 µg (load) 0.028 – 0.206 mg/mL R = 0.9998 Slope = 762455 Intercept = -16740	0.61 – 2.14 µg (load) 0.123 – 0.427 mg/mL R = 0.9995 Slope = 122243 Intercept = -9104	0.17 – 1.58 µg (load) 0.034 – 0.316 mg/mL R = 0.9997 Slope = 120022 Intercept = -5053
Method Precision (%RSD)	1.2	0.6	0.6	0.8
Specificity	No interference	No interference	No interference	No interference
Accuracy (%Recovery)	91.8	91.3	109.8	109.5
Detection Limit	Not evaluated	Not evaluated	Not evaluated	Not evaluated
Quantitation Limit	0.056 mg/mL	0.028	0.123	0.034

3.7.3. COMPARISON TO CURRENT TECHNOLOGY (CE) AND POTENTIAL APPLICATIONS

The use of 2D-LC for the separation and quantitation of free carrier protein and polysaccharide in glycoconjugate vaccines is a novel application. However, the technology must demonstrate acceptable performance prior to implementation in a vaccine development process. To evaluate the feasibility of 2D-LC, the method performance was compared to an established technology for this application. Capillary electrophoresis (CE) is a technology commonly utilized for the separation and quantitation of both free carrier protein and polysaccharide in glycoconjugate vaccines and seemed to be an appropriate choice for comparison. The CE methods for comparison separated and quantitated free carrier protein and polysaccharide using an SDS-modified buffer coupled with UV detection. Results for only CP8-CRM$_{197}$ samples were used in this comparison due to less free carrier protein and polysaccharide inherently present in

the sample from the manufacturing process compared to CP5-CRM_{197} samples. This provided a more representative assessment of analytical method performance near the lower range of the method e.g. quantitation limit.

The results from the qualification of the 2D-LC method (Section 3.7) and the validation from the two independent, established CE methods are summarized in Table 51. A difference was observed in accuracy, where CE showed to be more accurate across analyses of free carrier protein and polysaccharide. For this qualification, the theoretical amounts of free carrier protein and polysaccharide in the spike samples were based off results obtained from the CE methods. Inherent bias between technologies on the measurement of these components could lead to the differences observed in accuracy performance. The percent recoveries were within 10% for 2D-LC, which is acceptable for a quantitative residual impurity method. The 2D-LC method shows slightly higher precision compared to the CE methods. Overall, the results showed comparable performance between the two technologies.

Table 51. Qualification Summary of Free Carrier Protein and Free Polysaccharide in CP8-CRM$_{197}$ by Capillary Electrophoresis (CE) and 2D-LC

Parameter	Free Carrier Protein		Free Polysaccharide	
	CE	2D-LC	CE	2D-LC
Linearity Sample	0.025 – 0.130 mg/mL R = 0.9977 Slope = 146800 Intercept = -239	0.028 – 0.206 mg/mL R = 0.9998 Slope = 762455 Intercept = -16740	0.040 – 0.260 mg/mL R = 0.9995 Slope = 75347 Intercept = -65	0.034 – 0.316 mg/mL R = 0.9997 Slope = 120022 Intercept = -5053
Method Precision (%RSD)	1.6	0.6	1.1	0.8
Specificity	No interference	No interference	No interference	No interference
Accuracy (%Recovery)	100.6	91.3	99.7	109.5
Detection Limit	Not evaluated	Not evaluated	Not evaluated	Not evaluated
Quantitation Limit	0.025	0.028	0.040	0.034

Several advantages of CE are a high separation power, automated instrumentation, and the ability to analyze large molecules, such as glycoconjugate vaccines, without concern of clogging the inlet of the capillary. However, the CE technology can be susceptible to instrumentation robustness issues (e.g. capillary performance, instrumentation errors, matrix effects, etc.) and suffers from low sample throughput. These issues can have a significant impact on the efficiency of analysis. For example, the CE methods described above can analyze 10 to 13 samples over a period a two-day period. Whereas the 2D-LC method is capable of analyzing over 80 samples in the same amount of time. In addition, the 2D-LC includes the analysis of both free protein and polysaccharide in a single injection. This drastically reduces the amount of time and labor spent on sample analysis, resulting in an overall increase in sample throughput. Robust and efficient analysis of quality attributes is desired to help progress vaccine candidate in a research and development environment. Manufacturing process and formulation development

studies can generate many samples and often require quick and efficient samples analysis for decisions. As performance of the 2D-LC has been demonstrated to be comparable to current technologies with the potential for much higher throughput and robustness, it appears to be a viable alternative technology for implementation into the development process for glycoconjugate vaccines.

3.8. Application to Additional CRM_{197} Glycoconjugate Vaccines

The feasibility of applying this 2D-LC method to additional CRM_{197} glycoconjugate vaccines as a potential platform was evaluated. A method is considered "platform" when it can be successfully applied to a high percentage of molecules of the same type or modality. Using an 80:20 rule as a guideline is generally acceptable to consider a method platform, where the method works for 80% of the molecules evaluated. Platform methods can reduce time and resources in the method development process by providing a starting point for method conditions previously shown to work for similar molecules. Glycoconjugate vaccines are often multi-valent to target various serotypes most prevalent in nature or known to cause a high percentage of infections in the population. The chemistries for conjugating polysaccharide to the carrier protein are often shared between the serotypes. Therefore, the quality attributes of the glycoconjugate drug substance that must be monitored are typically shared across serotypes as well. Implementing a single technology or method to monitor a critical quality attribute is ideal. This reduces the complexity of the analytical testing by not having to invest in multiple technologies to support the vaccine development process. In addition, this streamlines analytical method validation activities by reducing the number of methods requiring validations saving time and resources.

The strategy for evaluating the feasibility of the 2D-LC method as a platform technology was to analyze multiple serotypes from additional glycoconjugate vaccines. Therefore, three pneumococcal serotypes (Pn) and one Group B streptococcus (GBS) serotype were used for analysis. The pneumococcal serotypes evaluated, 22F, TY8, and 33F, are of the same strain of bacteria, but have varying, unique structural characteristics, which can pose a challenge for any analytical method sensitive to these differences. In addition, the Group B streptococcus type III is a different strain of bacteria with its own unique structure. All serotypes evaluated have known structures published in literature [23-26]. For the purpose of these experiments, a qualitative approach was taken to assess the chromatographic separation of the components of interest. The evaluation of these serotypes provides insight on the capability of the 2D-LC method to be a platform technology by screening various serotypes expected within and across multi-valent glycoconjugate vaccines.

Purified polysaccharide and glycoconjugate reference material (provided in house) were used for analysis. The purified polysaccharide reference material for the various serotypes were first tested in a 1D format using the RPLC method conditions listed in Table 20. The purpose of this was to screen the polysaccharides and ensure they were retained, eluted, and detected, similarly to the *S. aureus* polysaccharides. This is critical as polysaccharides have hydrophilic character and can lack chromophores limiting the use of UV detection. Also, the added complexity of the 2D-LC system can convolute issues making trouble-shooting difficult. Purified polysaccharide reference standards were diluted to approximately 0.5 mg/mL in water and injected at 5 µL targeting an approximate 2.5 µg column load. This column load targeted the upper range evaluated for the *S. aureus* conjugates. An overlay from the RPLC analysis of the four polysaccharide serotypes is shown in Figure 67. The polysaccharide was only observed for the

Pn 22F serotype. Responses were observed near the void volume for all other serotypes and are likely from the polysaccharide. These observations were likely due to the hydrophilic character of polysaccharide resulting in poor retention on the column.

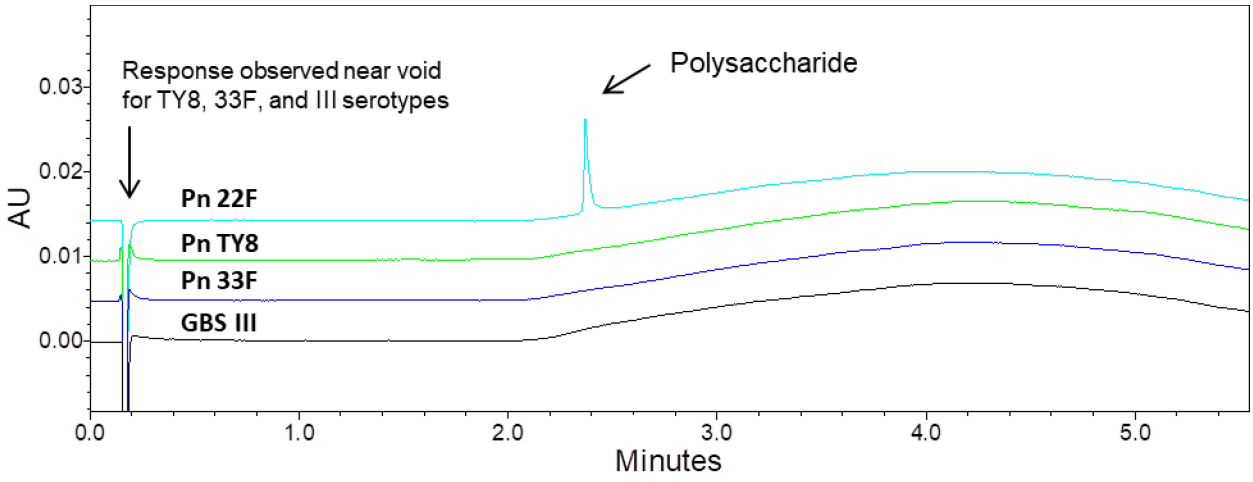

Figure 67 1D RPLC analysis of polysaccharide reference material from pneumococcal serotypes 22F, TY8, and 33F and Group B streptococcus serotype III. Polysaccharide peaks were only observed from 22F.

Retention of the polysaccharide was shown to be sensitive to slight changes in the starting percentage of mobile phase B (section 3.5.2). The experiment above was repeated after modifying the initial mobile phase conditions. The mobile phase percentage of B was reduced from 5% to 1% to increase retention of the polysaccharides. All other conditions remained the same. An overlay from the RPLC analysis of the polysaccharide serotypes is shown in Figure 68. All polysaccharide serotypes were retained, eluted, and detected within the expected retention time window. The polysaccharide peaks were observed to have differing retention times with the serotypes previously not observed having earlier retention times compared to Pn 22F. The three serotypes not initially observed are more hydrophilic and were not retained with the higher percentage of organic mobile phase. This shows the unique structural characteristics of each serotype impacts the interaction with the column stationary phase. In addition, the observation of the polysaccharide peaks showed that UV detection can be used for these

serotypes, but the peak responses varied significantly between serotypes. The GBS type III serotype showed a significantly greater response compared to the pneumococcal serotypes. This could be due to the polysaccharide repeat unit containing more highly substituted functional groups resulting in a greater UV response. This must be considered as it can impact detection and quantitation limits. The data from this experiment confirms serotype-dependent behavior in retention and detection attributed to structural differences between the serotypes. Overall, the updated method conditions proved successful in retaining and eluting the polysaccharides.

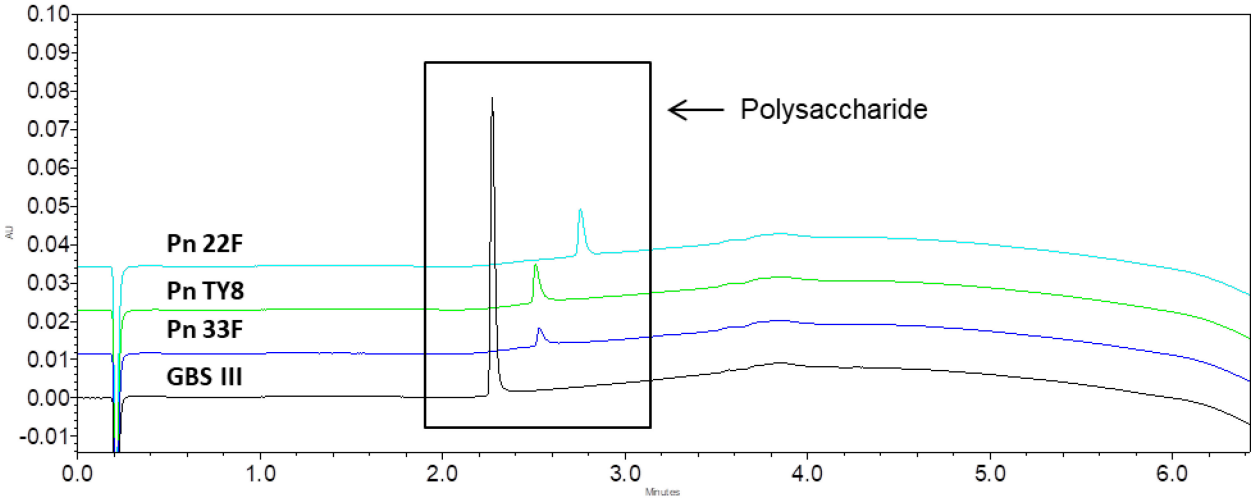

Figure 68 1D RPLC analysis of polysaccharide reference material from pneumococcal serotypes 22F, TY8, and 33F and Group B streptococcus serotype III using 1% mobile phase B at initial conditions. The polysaccharide peak was observed for all serotypes.

The additional serotypes were then evaluated in a 2D-LC format. The modified RPLC gradient conditions (with lower percent organic for the starting mobile phase) were applied to the 2D-LC (SEC-RPLC) method developed for the *S. aureus* glycoconjugate serotypes. For this evaluation, glycoconjugate samples were spiked with carrier protein and the respective polysaccharide reference material to generate a single glycoconjugate spike sample for each serotype. The target concentrations were approximately 0.1 mg/mL for free carrier protein and 0.5 mg/mL for free polysaccharide in the spiked glycoconjugate samples. This would allow for comparison

across serotypes as similar column loads were targeted. In addition, the respective polysaccharide reference materials were prepared to 1 mg/mL and analyzed to confirm polysaccharide peak retention times. Water was used a system blank. Samples were prepared and analyzed as per method. No quantitation was performed as the purpose of this experiment was to qualitatively evaluate the separation of the free carrier protein and polysaccharide components.

As the valve switch timing controls the transfer of peaks from the first dimension to the second dimension, it is a critical 2D-LC method parameter that must be considered when evaluating different serotypes or modalities. The valve switch is based on retention time, and differences in retention time can result in loss of recovery in the second dimension. Therefore, analyzing the polysaccharide reference material aided in identifying suitable valve switch timing. An overlay of the SEC analysis of the polysaccharide serotypes is shown in Figure 69 using UV detection at 214 nm. The retention time of the polysaccharides was approximately 2.75 minutes for all serotypes and similar to the *S. aureus* polysaccharides (Figure 51). This ensured the valve switch timing allowed the full peak to be captured within the sample loop during the transfer to the second dimension.

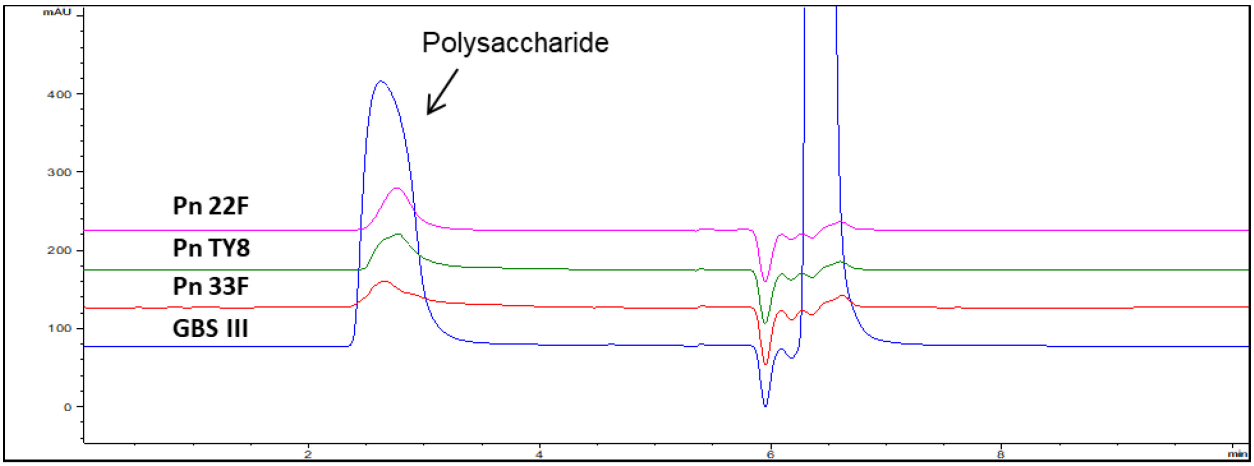

Figure 69 Overlay of the 1D SEC analysis of polysaccharide reference material from pneumococcal serotypes 22F, TY8, and 33F and Group B streptococcus serotype III. The valve switching time in the method was suitable as the retention time of polysaccharides were similar to the *S. aureus* polysaccharides.

The first dimension SEC analysis of the glycoconjugate spike samples for free carrier protein is shown in Figure 70 for the additional serotypes. The carrier protein peak was well resolved from the glycoconjugate peak as in the *S. aureus* serotypes. Variation in the glycoconjugate peak profiles were observed indicating differences in structure, heterogeneity, and size. The carrier protein peak, however, appeared to behave consistently across all serotypes. The carrier protein is within the calibration molecular weight range of the column, whereas the glycoconjugate is significantly larger and should elute near the exclusion void. Acceptable resolution of the glycoconjugate and the carrier protein peaks was achieved, and the method showed consistent performance across the additional serotypes evaluated.

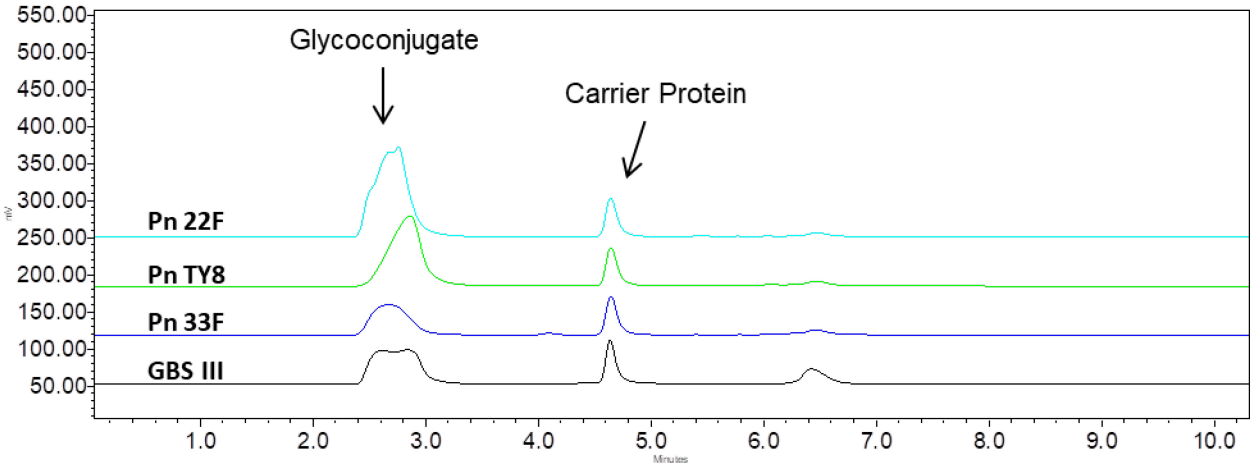

Figure 70 Overlay of the 1D SEC analysis of glycoconjugate spike samples from pneumococcal serotypes 22F, TY8, and 33F and Group B streptococcus serotype III. Acceptable resolution of the carrier protein from the glycoconjugate was achieved for all serotypes.

The second dimension RPLC analysis of the glycoconjugate spike samples for free polysaccharide is shown in Figure 71 for the additional serotypes. The respective purified polysaccharide reference materials were also analyzed along with the glycoconjugate spike samples to aid in peak identification. As previously observed in the 1D analysis of the polysaccharide reference material, the polysaccharide peak was not observed in the retention time window for all serotypes. Pneumococcal serotypes 33F and 22F were observed, and relative retention times of these serotypes were consistent with the previous 1D analysis. However, 22F showed an unusually low response. The 22F polysaccharide reference material showed no additional peaks when compared to the water blank. The lower response in the 2D-LC format is not fully understood and will require further investigation. No polysaccharide peak was observed for pneumococcal TY8 and Group B streptococcus type III serotypes. For the two serotypes where the polysaccharide was observed, adequate resolution of the polysaccharide from the glycoconjugate and carrier protein was achieved.

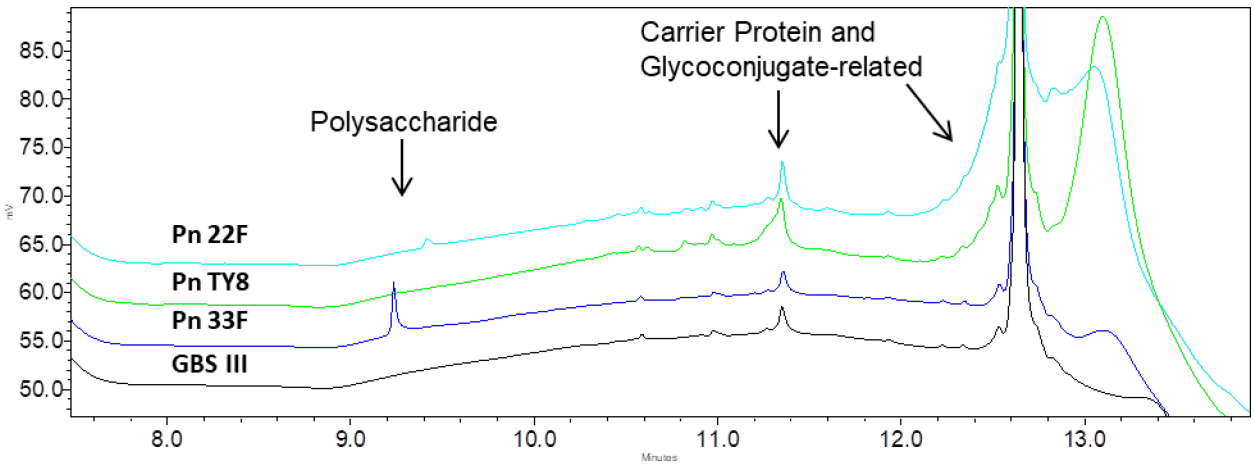

Figure 71 Overlay (zoom) of the 2D RPLC analysis of glycoconjugate spike samples from pneumococcal serotypes 22F, TY8, and 33F and Group B streptococcus serotype III. Polysaccharide peak was not observed for all serotypes tested. Acceptable resolution of the polysaccharide from the glycoconjugate and carrier protein peaks was achieved for observed serotypes.

The 2D RPLC data was further probed to identify any potential causes for the lack of polysaccharide peak in pneumococcal TY8 and Group B streptococcus III. The absence of the polysaccharide peak was thought to be due to weak retention, which has been observed in a previous experiment with these specific serotypes. A potential area of concern is the flow-through region during the transfer of the SEC mobile phase to the second dimension column. The peaks of interest are focused on the head of the column by hydrophobic interactions with the stationary phase, which allows the baseline to equilibrate from the disturbance of the transferred SEC mobile phase and reduces band broadening. The pneumococcal TY8 glycoconjugate spike sample was overlaid with its respective polysaccharide reference material and water (Figure 72). The injection of water was included as a system blank to aid the identification of sample-related peaks. Upon closer inspection of the flow-through region, a significant response was observed in both the glycoconjugate spike sample and the polysaccharide reference material but was not present in the water blank. As the response was present in the purified reference material and not in the water blank, it confirms the response is polysaccharide-related. Furthermore, the lack of polysaccharide peak in the expected retention

time window along with prior knowledge of the polysaccharide eluting near the void indicates the response is likely due to un-retained polysaccharide. The interaction of the polysaccharide with the salt and other components in the SEC mobile phase may be more favorable due to the hydrophilic and polar characteristics of the polysaccharide, preventing interaction with the hydrophobic stationary phase. This would result in even weaker retention and loss of polysaccharide peak recovery.

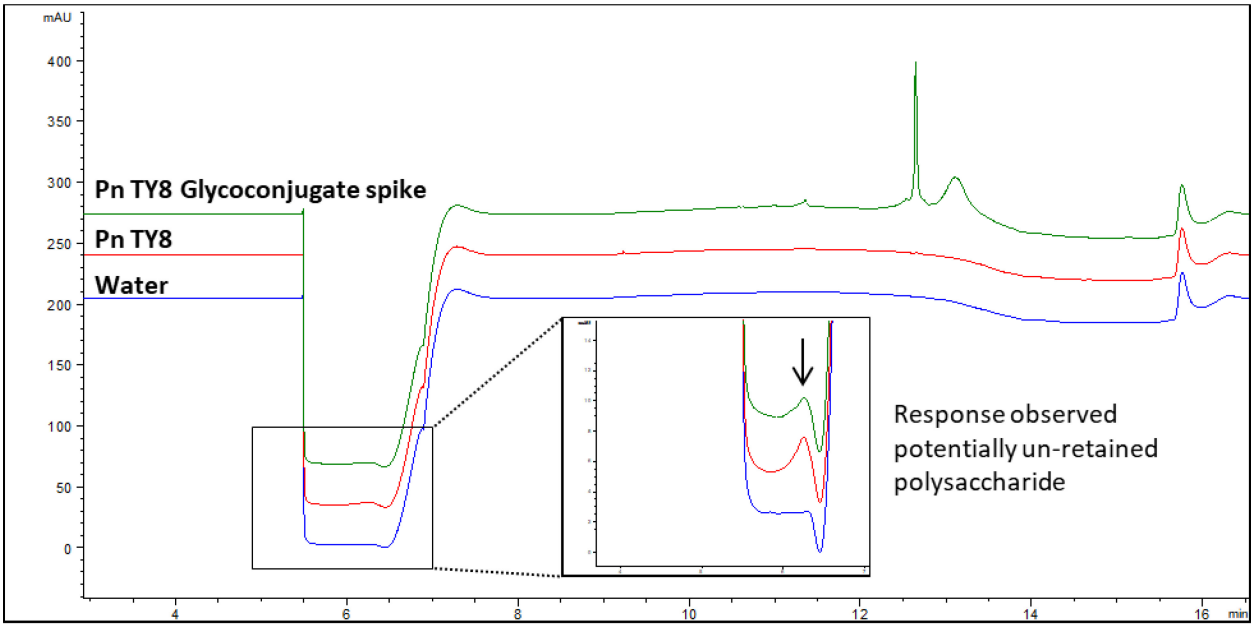

Figure 72 Overlay of the 2D RPLC analysis of TY8 glycoconjugate spike samples, TY8 purified polysaccharide, and water (blank). Response was observed in the SEC mobile phase flow-through for both polysaccharide and glycoconjugate spike samples indicating the peak is un-retained polysaccharide.

The same analysis performed for pneumococcal TY8 was applied to Group B streptococcus type III to probe any causes for the lack of polysaccharide peak. An overlay of the GBS type III glycoconjugate spike and its respective polysaccharide reference material is shown in Figure 73. Interestingly, the opposite result was observed for the GBS type III, where a peak was observed at the flow-through in the glycoconjugate spike and not the polysaccharide reference material. Additional information indicates the peak observed is likely attributed to the glycoconjugate

rather than the polysaccharide. Firstly, the GBS type III glycoconjugate peak in Figure 73 (retention time approximately 13.2 min.) shows a significantly lower response compared to the other serotypes evaluated, which could be from poor retention of the conjugate. And secondly, the peak should not be attributed to any small molecule matrix components present in the sample as they would not be transferred to the second dimension since small molecules elute in the inclusion void after the valve switch occurs. A further complicating observation is that the polysaccharide was not observed in any region of the chromatogram. This issue has not been resolved and will require further investigation and development to identify the cause.

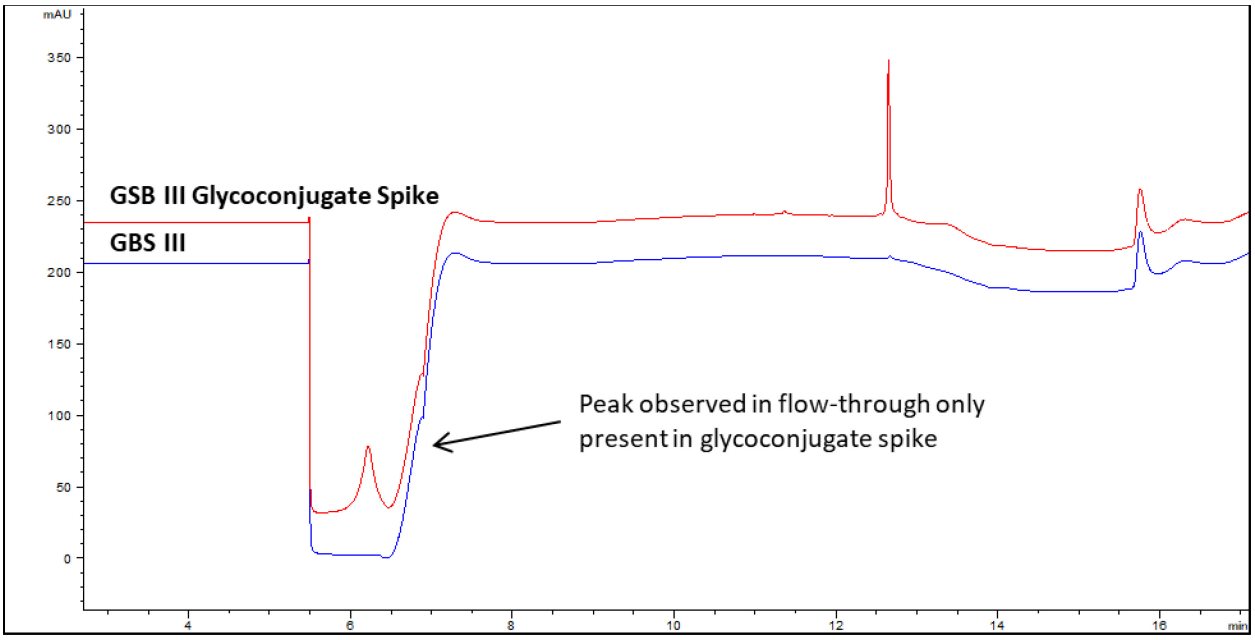

Figure 73 Overlay of the 2D RPLC analysis of GBS III glycoconjugate spike sample and GBS III purified polysaccharide. Response was observed in the SEC mobile phase flow-through for only the glycoconjugate spike sample indicating the peak is not likely polysaccharide-related.

In summary, this 2D-LC method was applied to additional glycoconjugate serotypes from pneumococcal and Group B streptococcus to evaluate its potential as a platform method for glycoconjugate vaccines. In the first dimension SEC analysis, the method proved successful as a platform method for free carrier protein. The RPLC performed as a platform method for the

serotypes evaluated for free polysaccharide in a 1D format. However, issues arose when applying the RPLC analysis for free saccharide to the second dimension, and mixed results were obtained. The polysaccharide was shown to have poor or no response in some serotypes, likely due to weak retention, poor UV response, or other unknown factors. The observed variability in polysaccharide behavior is largely attributed to the structural differences between serotypes. Therefore, the current 2D-LC method is not suitable as a platform method for glycoconjugate vaccines. Further development and method modifications for suitable chromatographic separation in the second dimension are required.

4. CHAPTER 5: CONCLUSION

Free carrier protein and polysaccharide are critical quality attributes of glycoconjugate vaccines that require monitoring during vaccine development, commercial release testing, and stability. The work presented here was aimed to the development of a novel analytical technique to address the inefficiencies of the current technology for measuring these attributes. Initial work focused on the development of a HIC method that utilized the separation space prior to the void volume for a unique sized-based separation of the polysaccharide. As glycoconjugates are complex sample mixtures, difficulties arose when trying to optimize resolution between the varying components. Improving the resolution of one component typically resulted in a decrease of another. Also, the hydrophobic properties of the carrier protein and glycoconjugate proved difficult to analyze due to very tight binding to the HIC stationary phases causing issues with elution. Therefore, the focus shifted to the development of 2D-LC method for this application. 2D-LC is useful for efficiently separating complex mixtures by using orthogonal modes of separation to achieve greater selectivity. The developed 2D-LC method demonstrated the capability of quantitating both free carrier protein and polysaccharide in two *S. aureus* glycoconjugate serotypes, CP5 and CP8, with a single injection. This effectively reduces the analyses of both attributes to a single method. Compared to analysis by CE, which is a commonly used technology for this application, HPLC can achieve a significantly higher throughput with more robust instrumentation. A qualification was performed as per ICH guidelines to assess the method performance, which was comparable to CE methods. With higher throughput and improved robustness, the method is a potentially valuable tool in the manufacturing process development where high sample loads often require quick and efficient sample analysis for process decisions. Though 2D-LC has made significant improvements in

instrumentation and software, a limitation of the technology is the validation of 2D-LC instrumentation, making the implementation into a quality control environment challenging. Finally, the 2D-LC method was applied to additional vaccines, such as pneumococcal and Group B streptococcus serotypes, for evaluation as a platform application across glycoconjugate vaccines. However, due to structural differences between the serotypes, performance varied across the serotypes evaluated. Further method modifications would be required for suitable chromatographic separation across multiple glycoconjugate vaccines. In summary, the work presents a novel and efficient tool for the analysis of free carrier protein and polysaccharide for *S. aureus* glycoconjugates.

5. APPENDICES

5.1. SEC MOBILE PHASES

Mobile phases used during the development of the SEC method for separation of free protein are listed in Table 52.

Table 52 Index of Mobile Phases Used during SEC Development

ID	Composition	Preparation Information
A	30 mM Sodium Phosphate, 150 mM Sodium Chloride, 0.1 mM EDTA, pH 6.7	Dibasic sodium phosphate heptahydrate and monobasic sodium phosphate monohydrate used; no titration
B	50 mM Sodium Sulphate, 20 mM Phosphate Buffer, pH 6.8	Anhydrous sodium sulphate and sodium phosphate monobasic monohydrate used; pH adjusted with NaOH
C	30 mM Sodium Phosphate, 300 mM NaCl, pH 6.7	Dibasic sodium phosphate heptahydrate and monobasic sodium phosphate monohydrate used; pH adjusted with NaOH
D	30 mM Sodium Phosphate, pH 6.7 in 5% IPA	Dibasic sodium phosphate heptahydrate and monobasic sodium phosphate monohydrate used; no titration prior to mixing with IPA
E	1X PBS, pH 7.2	Commercially available (Gibco). No preparation.

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