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A Systematic Optimization Analysis of N-Linked Glycans on Porous Graphitic Carbon (PGC) at Different Column Temperatures

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Abstract

Protein glycosylation is a post-translational modification where glycans are covalently attached to proteins. N-linked glycosylation is a type of protein glycosylation which involves the covalent attachment of an oligosaccharide to an asparagine residue within a polypeptide chain. Glycans are involved in a number of biological processes such as cell-cell interactions, cell adhesion, immune recognition, and protein folding and function. However, aberrant glycosylation is mostly associated with many types of diseases such as cancer, autoimmune disorders and many other diseases. Therefore, understanding the variations in glycomics profiles on protein has become very crucial for molecular testing. Despite the advance in LC-MS/MS for structural diverse N-glycan analysis, the separation and analysis of N-linked glycans are analytically challenging due to the molecule large number of possible isomeric conformations, difficult to ionize and contain a variety of complex composition, branching and linkage isomers. Even though, many reversed PGC phases have been used for the separation of N-glycans, especially in the context of LC-MS techniques but is still difficult to implement in a robust and reproducible manner. Herein, systematic optimization analysis of N-linked glycans in porous graphitic carbon (PGC) at different column temperatures was investigated. Underivatized tri-sialylated species and bi-sialylated species N-glycans derived from bovine fetuin and human transferrin respectively were utilized to optimize at different temperature. The study suggests that by optimizing at higher temperature, PGC columns can significantly enhance the resolution of complex glycan mixtures, offering powerful tools for in-depth glycomics optimization analysis.

1. Introduction

Protein glycosylation consists of oligosaccharides attached to asparagine residues. (Bapiro, T.E.; Richards, F.M. & Jodrel. D.I., 2016; Watanabe, Y. et al., 2021; Solá, R.J. & Griebenow, K., 2009) Protein glycosylation, specifically N-linked glycosylation, involves the covalent attachment of oligosaccharides to asparagine residues within a polypeptide chain. (Chandler, K.B. et al., 2019; Jansen, R.S.; Rosing,

H. et al., 2011) Glycosylation is one of the important protein post-translational modifications in eukaryotes. (Agrofoglio, L.A. et al., 2007) Glycans are involved in a broad range of biological processes such as cell-cell interactions, (Watanabe, Y. et al., 2021; Oyama, M. et al., 2018) cell adhesion, (Jansen, R.S. et al., 2011; Watanabe, Y. et al., 2021) immune recognition, (Bapiro, T.E.; Richards, F.M. & Jodrel. D.I., 2016; Shental-Bechor, D. & Levy, Y., 2008) host-pathogen interaction (Bapiro, T.E.; Richards, F.M. & Jodrel. D.I., 2016; Watanabe, Y. et al., 2021) and protein folding and function. (Shental-Bechor, D. & Levy, Y., 2008; Solá, R.J. & Griebenow, K., 2009) Aberrant glycosylation is mostly associated with many types of diseases such as cancer (Singh, C. et al., 2018), autoimmune disorders (Watanabe, Y. et al., 2021; Singh, C. et al., 2018) and traumatic brain injury (TBI) (Oyama, M. et al., 2018). Increase branching or alteration in complex N-linked glycans structures which often lead to the overexpression of truncated or incomplete glycans and the appearance of novel glycan structures is one of the most common glycosylation changes observed in cancer cells (Agrofoglio, L.A. et al., 2007). Therefore, understanding the variations in glycomics profiles on protein has become very crucial for molecular testing.

The separation and analysis of glycoprotein released N-linked glycans using LC-MS/MS techniques is substantially a challenging analytical task due to the molecule large number of possible isomeric conformations, difficult to ionize and contain a variety of complex composition, branching and linkage isomers (Bapiro, T.E.; Richards, F.M. & Jodrel. D.I., 2016; Shental-Bechor, D. & Levy, Y., 2008; Singh, C. et al., 2018; Ozdilek, A. et al., 2020). Despite the advance in LC-MS/MS techniques for structural diverse N-linked glycan analysis especially in sensitivity and specificity (Pandey, V.K. et al., 2022; A. Mehta, A.; Herrera, H. & Block, T., 2015), glycan labeling and derivatization techniques including Rapi-Fluor-MS (RFMS) and permethylation (Huang, Y. et al., 2017). Glycan derivatization can present several challenges basically due to inherent structural complexity with poor ionization efficiency (B.G. Cho, L. Veillon & Y. Mechref, 2019), longer sample preparation time (Reyes, C.D.G. et al., 2022; Zhou, S. et al., 2017), and unwanted side reactions (Palmisano, G. et al., 2013; Zhou, S. et al., 2017). To overcome these limitations, developing efficient chromatographic techniques for N-glycans analysis is necessary.

Hydrophilic interaction liquid chromatography (HILIC) (Melmer, M. et al., 2011), reversed-phase high-performance liquid chromatography (RP-HPLC) (Wuhrer, M.; Boer, A.R. & Deelder, A.M., 2009), mesoporous graphitized carbon (MGC) (Melmer, M. et al., 2011) and porous graphitized carbon (PGC) (Pereira, L., 2008) are some of the frequently used chromatographic techniques coupled with MS for glycans profiling. Despite the superb sensitivity of these procedures, differentiating between similar glycan structures pose a serious challenge because of the structural complexity and diversity of glycans (Wuhrer, M.; Boer, A.R. & Deelder, A.M., 2009; Palmisano, G. et al., 2013). Even though, porous graphitized carbon liquid chromatography (PGC-LC) coupled with MS has been reported to be a superb method for comprehensive analysis of N-linked glycans, (Pereira, L., 2008) but is still difficult to implement in a robust and reproducible manner due to the complexity and heterogeneity of glycans, especially N-linked glycans with multiple antennae, LacNac repeats and sialic acids. Herein, to overcome these limitations, the optimization analysis of N-linked glycans in porous graphitic carbon (PGC) at different column temperature was investigated. Underivatized tri-sialylated species and bi-sialylated species N-glycans derived from bovine fetuin and human transferrin respectively were utilized to optimize at different column temperature.

2. Materials and Method

2.1 Materials

Synthesized N-glycan standards (SRM 3655), 4 N-acetylglucosamine (HexNAc) units, 5 hexose (Hex) units, and 2 N-acetylneuraminic acid (NeuAc) units isomers were obtained from the National Institute of Standards and Technology (NIST) US Department of Commerce. Recombinant PNGase F was used for N-glycan release was procured from Promega Corporation (Madison, WI). alpha-2-HS-glycoprotein and beta-1 globulin protein, human blood serum (HBS), and borazane complex were acquired from R&D Systems (Minneapolis, MN, USA). Ammonium hydroxide was acquired from KMG Chemical Inc. (Texas, USA). HPLC-grade water,

acetonitrile, formic acid, ammonium formate and HPLC-grade methanol were obtained from were acquired from Sigma-Aldrich (St Louis, MO). Porous graphitized carbon (PGC) solid-phase extraction cartridges were used for sample cleanup. LC-MS analysis was performed using a porous graphitized carbon column (100 × 2.1 mm, 2.7 μm particle size, 200 Å pore size; Supel™ Carbon LC, Sigma-Aldrich, coupled to a high-resolution mass spectrometer (Orbitrap Exploris 240).

2.2 Sample Preparation

Synthesized N-glycans isomers of 4 N-acetylglucosamine (HexNAc) units, 5 hexose (Hex) units, and 2 N-acetylneuraminic acid (NeuAc) units were directly reduced with 10 μl of (10 μg/μl) of ammonia borane solution for 2 h at 65 °C. superfluous borane was changed into $[BO3]^{3-}$ by the addition of CH_3OH and eliminated by evaporation in a vacuum concentrator. N -linked glycans were released from 100 μg of standard glycoproteins, 50 μl of cerebrospinal fluid, and hemoglobin S (HbS) by first denaturing the glycoproteins at 90 °C for 30 min in the presence of G2 buffer, following established protocols for enzymatic deglycosylation using PNGase F with minor adaptations from published methods. (Gautam, S. et al., 2021) Proteins were denatured in 50 mM ammonium bicarbonate, optionally reduced and alkylated, and digested with trypsin to generate glycopeptides. The glycopeptides were desalted using C18 solid-phase extraction (SPE), and N-glycans were enzymatically cleaved with PNGase F at 37 °C overnight. Released glycans were separated from peptides using C18 SPE, lyophilized, and derivatized with procainamide via reductive amination (DMSO/acetic acid/water, sodium cyanoborohydride) at 65 °C overnight. Labeled glycans were desalted using PGC SPE, dried, and reconstituted in the initial LC mobile phase prior to analysis.

2.3 PGC Column Conditions

All samples were separated on a Porous graphitized carbon (PGC) column (100 × 2.1 mm, 2.7 μm, 200 Å; Supel™ Carbon LC, Sigma-Aldrich) coupled to a high-resolution mass spectrometer (Orbitrap Exploris 240). For mobile phase A (MPA), water with 10 mM ammonium formate (pH adjusted to ~7.5 with formic acid) was used while mobile phase B (MPB) contained acetonitrile with 10 mM ammonium formate. A multistep mobile phase gradient was applied: 0-5 min: 2% B (equilibration); 5-65 min: linear gradient 2→40% B; 65-75 min: 40→90% B (wash) and 75-90 min: re-equilibration at 2% B. The flow rate was 0.20 mL/min while temperature series were 25, 50, 75, and 100°C and the injection volume was 1-5 μL. Mass spectrometric detection was performed in positive electrospray ionization mode with a mass range of m/z 300-2000. Resolution was set to 60,000 (at m/z 200). Spray voltage was maintained at 3.5 kV with capillary temperature at 275°C. Data acquisition consisted of full scan MS with extracted-ion chromatograms (XICs) generated using ±5 ppm mass tolerance.

2.4 Data Analysis

Data (XICs) were generated for each glycan composition using Skyline and Mass-Hunter software. Peak integration was performed automatically with manual curation where necessary. Relative peak areas were calculated as (individual isomer peak area / total peak area for composition) × 100. Temperature-dependent changes in peak resolution, retention time, and relative abundances were assessed across the tested temperature range.

3. Results and Discussion

3.1 Workflow Description

The chromatographic signals of standard N-linked glycans enzymatically released from bovine fetuin and human Transferrin were initially evaluated and optimized to enhance their chromatography separation on a PGC column. The separated N-linked glycan isomers from alpha-2-HS-glycoprotein, beta-1 globulin protein, cerebrospinal fluid and human blood serum (HBS), samples were then analyzed using the optimal conditions.

3.2 Systematic Optimization

3.2.1 *Optimization of Sialylated N-glycans from Bovine Fetuin Across Different Column Temperatures*

This Figure 1a and 1b shows the behavior of sialylated N-glycans from bovine fetuin across different column temperatures. The column temperature was optimized using 25 °C, 50 °C, 75 °C and 100 °C. At lower temperatures (25-50°C), several glycan species were distinguishable, notably A3G3 (a tri-antennary, tri-galactosylated glycan). As the temperature increased, chromatographic resolution deteriorated: peaks representing highly

sialylated species became broader, overlapped, or were poorly retained. This suggests that the interactions governing PGC retention for negatively charged, sialylated glycans, primarily hydrogen bonding and electrostatic forces, are thermally sensitive (Chandler, K.B. et al., 2019; Agrofoglio, L.A. et al., 2007). Higher temperatures weaken these interactions, resulting in reduced retention strength and increased peak coalescence. Furthermore, the lability of terminal sialic acids under high temperature and solvent exposure may contribute to the diminished peak signals, although desialylation is unlikely under these mild conditions (Chandler, K.B. et al., 2019; Singh, C.; Shyanti, R.K. et al., 2018). These findings demonstrate that while neutral glycans benefit from higher temperatures, complex sialylated species require lower temperatures to maintain resolution on PGC columns (Chandler, K.B. et al., 2019; Agrofoglio, L.A. et al., 2007; Singh, C.; Shyanti, R.K. et al., 2018). Separation of highly sialylated species diminished at higher temperatures; A3G3 remained partially resolvable.

Mass-hunter analysis confirms the same trend observed in Skyline. Lower temperatures promote some degree of separation among sialylated glycan species, with A3G3 consistently observable. At elevated temperatures, the chromatographic performance diminishes significantly, particularly for multiply sialylated structures. These consistent results reinforce the conclusion that the retention of sialylated N-glycans on PGC is compromised by thermal weakening of non-covalent interactions (Chandler, K.B. et al., 2019; Agrofoglio, L.A. et al., 2007). A3G3 remains detectable, possibly due to its intermediate size, abundance, and balance between branching and charge, allowing it to retain PGC affinity across a broader temperature range. Figures 1a and 1b highlight the need for temperature optimization in glycomics workflows depending on the degree of glycan sialylation.

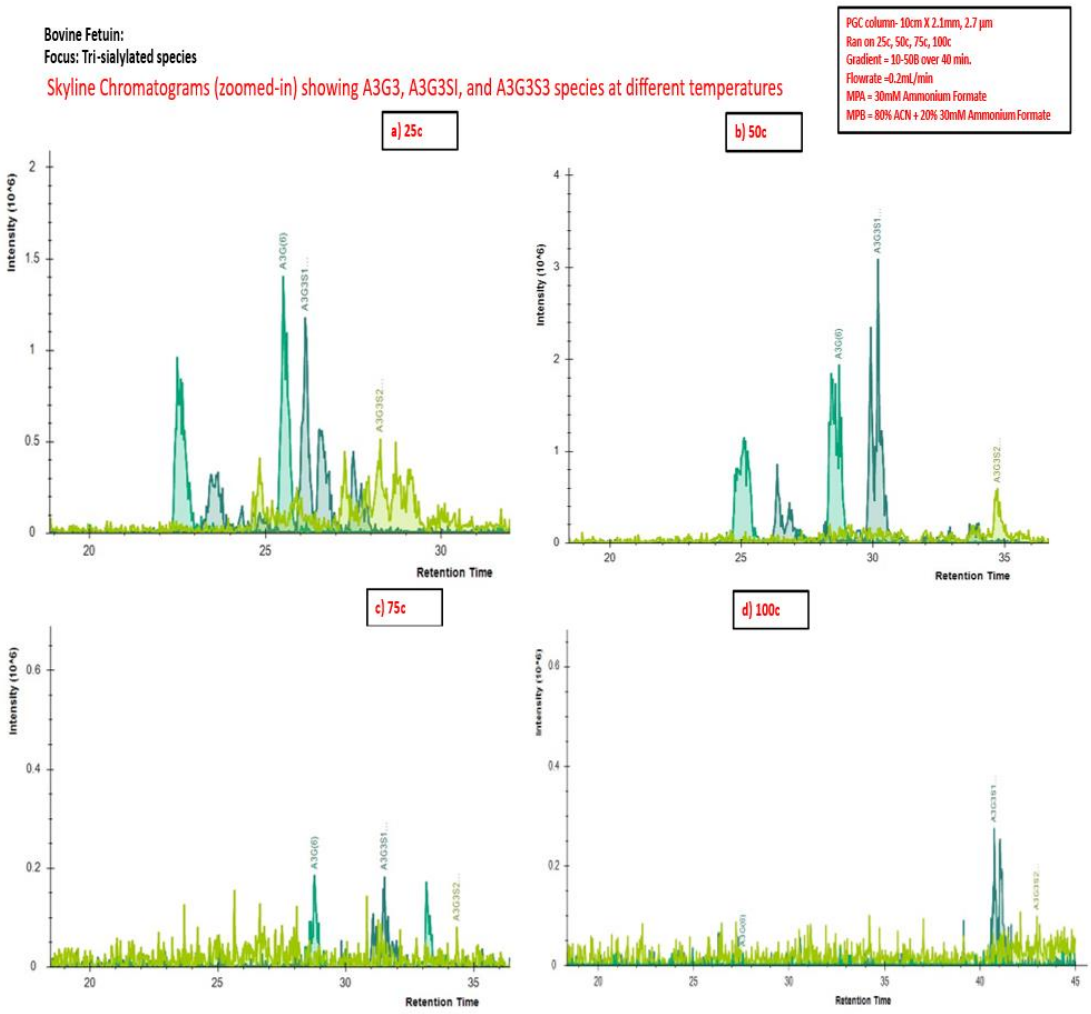

Figure 1a. Skyline chromatograms of bovine fetuin N-glycans at different column temperatures

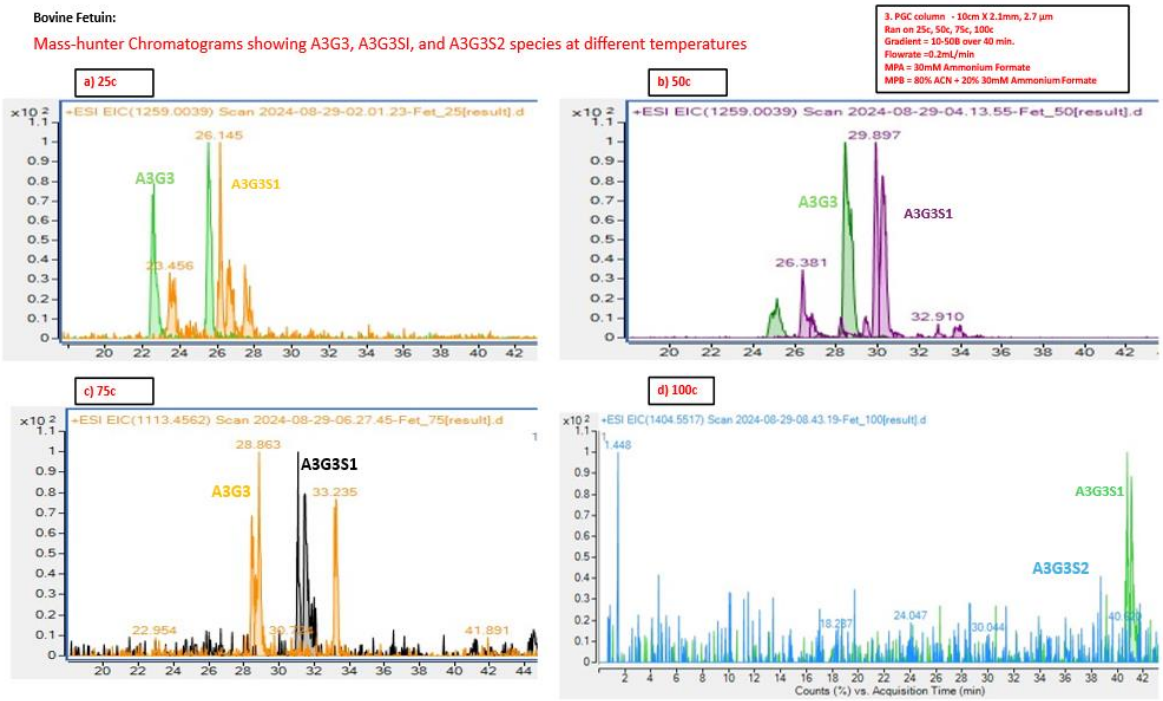

Figure 1b. Mass-hunter chromatograms of bovine fetuin N-glycans at different column temperatures

3.2.2 Optimization of Sialylated N-glycans from Human Transferrin Across Different Column Temperatures

Figures 2a and 2b revealed the N-glycan profile of human transferrin was dominated by bi-sialylated species, which were the most abundant glycans detected and thus served as the main reference for assessing temperature effects on separation. Under the same chromatographic parameters, increasing temperature produced similar effects to those observed with bovine fetuin, peak definition decreased at higher temperatures, and peak splitting occurred in certain bi-sialylated species. These splits likely represent structural isomers or linkage variants that were only partially resolved within the tested temperature range.

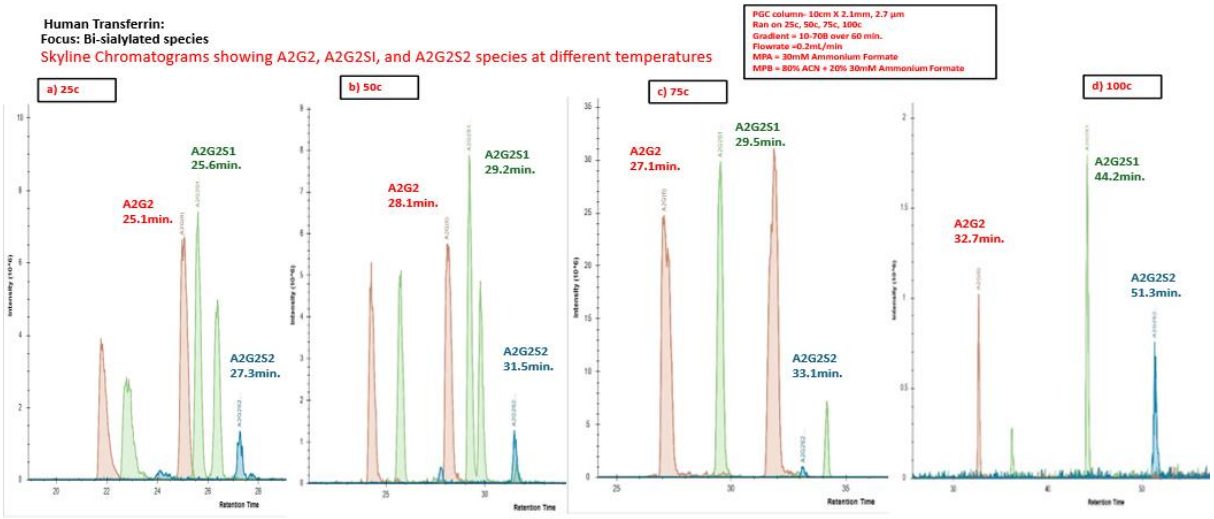

Figure 2a. Skyline chromatograms of Human Transferrin N-glycans at different column temperatures

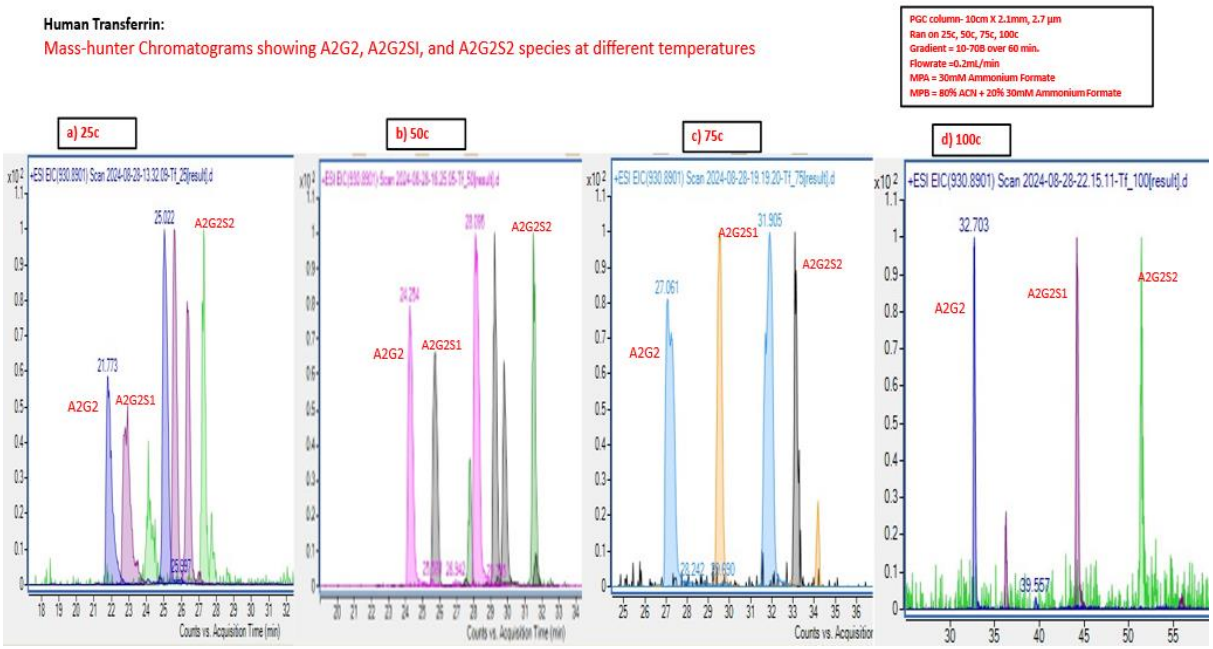

Figure 2b. Mass-hunter chromatograms of Human Transferrin N-glycans at different column temperatures

4. Conclusion

The study was on systematic optimization analysis of N-linked glycans on Porous Graphitic carbon (PGC) at different column temperatures. The improved chromatographic resolution for N-linked glycan was achieved by optimizing at different column temperatures. The N-linked glycans profile of bovine fetuin and human transferrin are dominated by tri-sialylated species and bi-sialylated species respectively are most abundant structures in the samples and therefore the primary focus for temperature-dependent systematic optimization analysis. At 25 °C, these glycans were partially resolved into multiple chromatographic peaks for the same m/z composition, indicating separation of structural isomers. As temperature increased to 50, 75, and 100 °C, peak broadening and coelution became more pronounced for the larger tri-sialylated species, reducing isomeric resolution. This suggests that elevated temperature weakens hydrogen bonding and electrostatic interactions between sialic acids and the PGC surface. Both Skyline and Mass-hunter visualizations confirmed the presence of multiple isomeric peaks and their temperature-dependent resolution. It was concluded that by optimizing at higher temperature, PGC columns can significantly enhance the resolution of complex glycan mixtures, offering powerful tools for in-depth glycomics optimization analysis

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