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## **Unravelling the sugar-coating of prostate-specific antigen : method development and its application to prostate cancer research**

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# HIGHLY SENSITIVE CE-ESI-MS ANALYSIS OF *N*-GLYCANS

FROM COMPLEX BIOLOGICAL SAMPLES

Based on Guinevere S.M. Lageveen-Kammeijer, Noortje de Haan, Pablo Mohaupt, Sander Wagt, Mike Filius, Jan Nouta, David Falck, Manfred Wuhrer, "*Highly Sensitive CE-ESI-MS Analysis of N-glycans from Complex Samples*", Submitted



## ABSTRACT

The in-depth characterization of released *N*-glycans remains a challenging task from low abundant proteins, limited sample amounts and of minor glycoforms in complex mixtures. Both high sensitivity and differentiation of isomeric *N*-glycan structures are required in biomarker research focused on protein glycosylation. A suitable approach for the highly sensitive and in-depth analysis of *N*-glycans is the hyphenation of capillary electrophoresis (CE) to mass spectrometry (MS), via electrospray ionization (ESI). However, *N*-glycans are either neutral (non-sialylated) or negatively charged (carrying one or several sialic acids), which hampers their efficient separation by CE and results in ionization biases during MS detection. Here, we present an easy workflow for the neutralization of sialic acids, which additionally enabled the differentiation between  $\alpha$ 2,3- and  $\alpha$ 2,6-linked sialic acids by MS based on their derivatized mass. Furthermore, an uniform charge was introduced to all *N*-glycans via reducing end tagging with a cationic hydrazide (Girard's reagent P). The sensitivity of the CE-ESI-MS method was optimized by the implementation of dopant enriched nitrogen gas to aid the ionization for MS detection. A highly sensitive detection of *N*-glycans was achieved with the presented platform in positive mode MS. Studying the total human plasma protein *N*-glycome, 167 glycoforms were identified, including sialic acid linkage-isomers. Being at least 100 times more sensitive than conventional matrix-assisted laser/desorption ionization time-of-flight (MALDI-TOF)-MS methods, CE-ESI-MS provides a highly complementary tool in the field of *N*-glycan analysis.





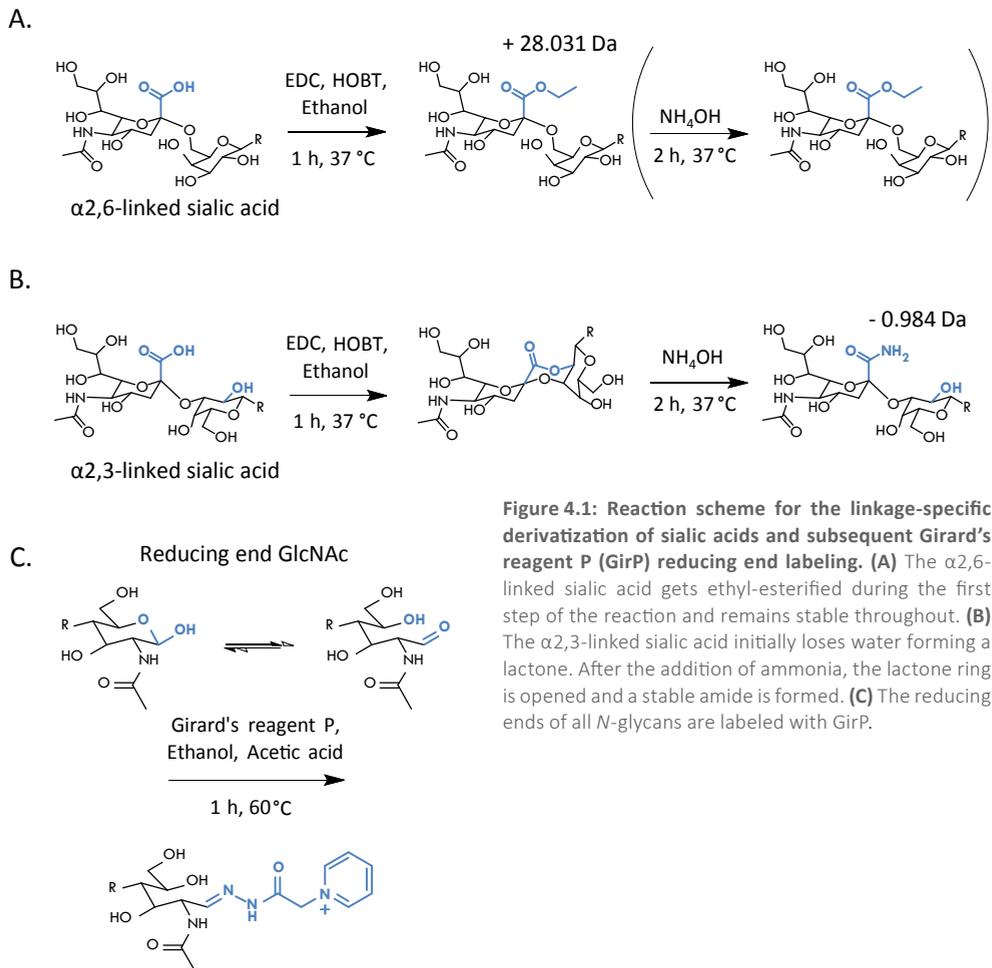
## INTRODUCTION

Glycosylation is known as one of the most complex post-translational protein modifications and is involved in many biological processes, including protease binding and antibody-receptor interactions.<sup>82</sup> In addition, the structure of a protein, as well as its solubility, are largely influenced by its glycosylation<sup>82</sup> and alterations in protein glycosylation have been associated with diseases such as rheumatoid arthritis and cancer.<sup>3,212,213</sup> Furthermore, the efficacy of biopharmaceuticals, and their half-life, is highly affected by associated glycosylation features.<sup>149-151</sup> Therefore, it is of utmost importance to have instruments that can characterize the glycosylation of a protein, or a set of proteins, in an in-depth manner. However, differentiating glycan isomers and obtaining information about monosaccharide linkage-positions remains a challenging task. Of particular importance is the differentiation of *N*-acetylneuraminic acids (further referred to as sialic acids) linked to galactoses ( $\alpha$ 2,3 or  $\alpha$ 2,6), as these are implicated in different biological processes, such as protein clearance (protein half-life) and glycan interaction with glycan-binding proteins such as galactins.<sup>8,9,144</sup> In addition, the differently linked sialic acids have biomarker potential for a range of diseases.<sup>92,97</sup> For example, in the case of prostate cancer, where it has been suggested that the sialic acid linkage-isomers present on prostate-specific antigen (PSA) provide a better sensitivity and specificity in the early detection of this type of cancer than serum PSA concentration alone.<sup>33-35,214</sup> Besides the need for a platform that can provide extensive structural information about protein glycans, there is also a great demand for more sensitive techniques to perform glycosylation analysis on minimal amounts of sample and (glyco) proteins that are only present in low concentrations.<sup>107,215</sup>

Capillary electrophoresis (CE), hyphenated to mass spectrometry (MS) via electrospray ionization (ESI), provides an attractive approach to characterize *N*-glycans released from proteins, as it allows highly sensitive detection as well as a good separation efficiency.<sup>216-218</sup> However, released *N*-glycans are not uniformly charged, as they might carry either zero, one or multiple negatively-charged sialic acids. This hampers the analysis by CE-ESI-MS in multiple ways, namely by the introduction of an ionization bias during MS detection, by the migration of the sialylated species towards the inlet in normal mode CE (detection at the cathodic end of the capillary), and by compromising the separation of the neutral (non-sialylated) species in both normal and reversed mode CE separation, as the migration will only be driven by the electro-osmotic flow (EOF). Previous work showed that the labeling of *N*-glycans at their reducing end with a negatively charged label, like 8-aminopyrene-1,3,6-trisulfonic acid (APTS) or 8-aminoapthalene-1,3,6-trisulfonic acid (ANTS), enables the simultaneous and efficient separation of both sialylated and non-sialylated glycoforms by reversed mode CE.<sup>217,219</sup> The combination of sialic acid neutralization and the introduction of an uniform label to the *N*-glycans results in the same charge for all species, enabling separation based on the



hydrodynamic volume only. For example, sialic acid neutralization and positive labeling of the *N*-glycan reducing end by aminoxy-tandem mass tag (TMT) provided the opportunity to separate and detect *N*-glycans in an efficient manner using normal mode CE and positive mode MS.<sup>220</sup> In addition, the linkage-specificity of the sialic acid neutralization in the latter approach (either with APTS or TMT tag), resulted in the differentiation between  $\alpha$ 2,3- and  $\alpha$ 2,6-linked sialic acids by MS.<sup>216,221</sup> Linkage-specific sialic acid derivatization can be performed via both esterification and amidation reactions,<sup>155,160,222-224</sup> often leading to lactone formation for the  $\alpha$ 2,3-linked sialic acids. However, lactones are prone to hydrolysis during subsequent sample preparation steps at elevated temperatures, generating unwanted underderivatized  $\alpha$ 2,3-linked sialic acids.<sup>160,221,222,224</sup>



**Figure 4.1: Reaction scheme for the linkage-specific derivatization of sialic acids and subsequent Girard's reagent P (GirP) reducing end labeling. (A)** The  $\alpha$ 2,6-linked sialic acid gets ethyl-esterified during the first step of the reaction and remains stable throughout. **(B)** The  $\alpha$ 2,3-linked sialic acid initially loses water forming a lactone. After the addition of ammonia, the lactone ring is opened and a stable amide is formed. **(C)** The reducing ends of all *N*-glycans are labeled with GirP.

In this study, the use of CE-ESI-MS was explored for an in-depth analysis of released *N*-glycans by combining linkage-specific sialic acid derivatization with the introduction of a permanent cationic label at the *N*-glycan reducing end. To overcome the instability of the lactonized  $\alpha$ 2,3-linked sialic acids during reducing end labeling, a simple additional step was implemented turning lactones into amide derivatives, while keeping the ethyl esters on the  $\alpha$ 2,6-linked sialic acids intact (**Figure 4.1.A** and **4.1.B**). For the reducing end labeling, a low-cost, readily available hydrazide label, Girard's reagent P (GirP), was employed (**Figure 4.1.C**).<sup>225</sup> To enhance the sensitivity of the CE-ESI-MS platform for the analysis of the *N*-glycan derivatives, dopant enriched nitrogen (DEN)-gas was implemented.<sup>226</sup> The sensitivity of the current CE-ESI-MS method was assessed and compared to a frequently used matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF)-MS platform. Furthermore, using this novel CE-ESI-MS(/MS) approach, the total human plasma protein *N*-glycome (TPNG) was studied, resulting in the identification of 167 *N*-glycan compositions, with differentiation of sialic acid linkage-variants.



## MATERIALS AND METHODS

The materials used in this research can be found in **Section S-4-1-1, Supporting Information**. Additional details on the *N*-glycan release, the optimization of linkage-specific sialic acid neutralization by ethyl esterification and amidation (EEA), sialic acid derivatization by double amidation (DA)<sup>222</sup> and the purification of *N*-glycans and sialyllactose standards can be found in **Section S-4-1-2 - S-4-1-4, Supporting Information**.

### PERMANENT CATIONIC LABELING OF THE *N*-GLYCAN REDUCING END

After sialic acid derivatization, either by EEA or DA, released *N*-glycans were labeled at the reducing end with GirP. Initial reaction conditions were as follows, 5  $\mu$ L of sialic acid-derivatized *N*-glycans, purified by hydrophilic interaction chromatography (HILIC), were mixed with 95  $\mu$ L GirP reagent (15 mM GirP in 85% ethanol (EtOH), 10% acetic acid (HAc) and 5% ultrapure deionized water (MQ)) and incubated for 2 h at 60°C. After incubation, 105  $\mu$ L acetonitrile (MeCN) was added and cotton HILIC solid phase extraction (SPE) was performed as described in **Section S-4-1-3, Supporting Information**, using 85% MeCN washing solutions and 10  $\mu$ L elution volume. Optimization of the protocol included decreasing the percentage of MQ in the reagent, increasing the concentration of GirP, decreasing the sample volume, decreasing the reagent volume and decreasing the incubation time (**Section S-4-1-5** and **Table S-4.1, Supporting information**). The optimized reaction conditions included a drying step of the *N*-glycan sample prior to adding 2  $\mu$ L of GirP reagent (50 mM GirP in 90% EtOH and 10% HAc) and 1 h incubation at 60°C. After incubation, the samples were dried by vacuum concentration at 60°C and dissolved in 10  $\mu$ L MQ for CE-ESI-MS analysis or 33.3  $\mu$ L 85% MeCN for HILIC purification and MALDI-TOF-MS analysis.

## SENSITIVITY ASSESMENT FOR MALDI-TOF-MS AND CE-ESI-MS METHODS

Two glycan standards (H3N4 and H5N4) were dissolved in water and mixed in a final concentration of 3333 fmol/ $\mu$ L for each glycoform. The mixture was diluted 10, 20, 100, 200, 1,000, 2,000 and 10,000 times and 6 replicates of 3  $\mu$ L of each of the dilutions (10,000, 1,000, 500, 100, 50, 10, 5 and 1 fmol of each glycan) were subjected to EEA and HILIC purification, and eluted in 10  $\mu$ L MQ (**Table S-4.2, Supporting information**). Three of the replicates were analyzed by MALDI-TOF-MS. The remaining three replicates were dried, labeled with GirP following the optimized protocol as described above and analyzed by CE-ESI-MS.

Procedures for the MALDI-TOF-MS and CE-ESI-MS analysis are described in **Section S-4-1-7** and **S-4-1-8, Supporting Information**, respectively. In addition, **Section S-4-1-9, Supporting Information** describes the data processing of MALDI-TOF-MS and CE-ESI-MS data using MassyTools<sup>202</sup> and LacyTools<sup>162</sup>, respectively.

# RESULTS AND DISCUSSION

## LINKAGE-SPECIFIC SIALIC ACID NEUTRALIZATION

It is known that *N*-glycan conjugates with differentially linked sialic acids can be separated without any further modification of the analytes by CE,<sup>6</sup> porous graphitized carbon liquid chromatography (LC)<sup>165</sup> or ion mobility MS.<sup>154</sup> However, during this study it was chosen to neutralize the sialylated species in a linkage-specific way, in order to analyze them simultaneously with the non-sialylated species in normal mode CE and to simplify their identification in MS(/MS). A variety of methods are known for the linkage-specific neutralization of sialic acids, resulting in either lactonization,<sup>160,224</sup> amidation<sup>222</sup> or methyl amidation<sup>223,227</sup> of the  $\alpha$ 2,3-linked sialic acids and methyl esterification,<sup>160,224</sup> ethyl esterification,<sup>160</sup> isopropylamidation<sup>223</sup> or dimethylamidation<sup>155,222</sup> of  $\alpha$ 2,6-linked sialic acids. Approaches resulting in (methyl)amidation of the  $\alpha$ 2,3-linked sialic acids are preferred over those inducing lactone formation, as the latter is prone to hydrolysis, especially during further processing or storage of the samples.<sup>221,222</sup> Most of these two-step approaches need a cleanup-step in between, to ensure stability as well as the differentiation of the differently linked sialic acids.<sup>223,227</sup> However, this extra cleanup is prone to (biased) sample loss, making it preferable to have a one-pot sample preparation, avoiding intermediate cleanup while keeping the required two-step reaction.<sup>222</sup> In order to generate a derivative of the  $\alpha$ 2,3-linked sialic acids with long-term stability, we introduced an amidation step using ammonia after the ethyl esterification ( $\alpha$ 2,6-linked sialic acids) and lactonization ( $\alpha$ 2,3-linked sialic acids) step of the original ethyl esterification protocol.<sup>160</sup> The ammonia step turned the lactonized  $\alpha$ 2,3-linked sialic acids into amides (290.111 Da), while keeping the ethyl esters on  $\alpha$ 2,6-linked sialic acids intact (319.127 Da; **Figure 4.1.A** and **4.1.B**). The method was optimized

using sialyllactose standards and optimization was further monitored using 23 *N*-glycan compositions of the TPNG (**Figure S-4.1, Supporting information**). The optimal concentration of ammonium hydroxide (NH<sub>4</sub>OH) in the second step of the reaction was found to be 1.15 M, resulting in 100.0% ± 0.02% (± standard deviation) of amide formation and 99.1% ± 0.3% of ethyl ester formation for 3'-sialyllactose and 6'-sialyllactose, respectively (*m/z* 775.286 and 804.301; **Figure S-4.1.A and B, Supporting information**). The reaction specificity was highly comparable to the specificity reported for ethyl esterification alone (above 97% specificity for both sialyllactoses),<sup>160</sup> while higher concentrations of NH<sub>4</sub>OH (1.60 or 1.98 M) resulted in a minimum specificity of 94.4% ± 3.0% on 6'-sialyllactose. When applying the optimized reaction conditions (using a final concentration of 1.15 M NH<sub>4</sub>OH; EEA) on TPNG *N*-glycans, the profiles obtained were highly comparable to those resulting from the original ethyl esterification protocol (**Figure S-4.1.C, Supporting information**). The most intense signal resulted from a diantennary *N*-glycan with two α2,6-linked sialic acids and without core-fucose (H5N4F1S<sub>2</sub>, *m/z* 2301.835)<sup>160</sup> with a relative intensity of 54.0% ± 4.0% after EEA and 56.3% ± 1.3% after ethyl esterification. The EEA conditions were furthermore compared to the DA conditions for released *N*-glycans as described previously,<sup>222</sup> which resulted in highly comparable TPNG profiles, showing the relative abundance of the highest signal to be 53.4% ± 1.0% and 54.3% ± 0.6% for the DA and EEA, respectively (**Figure S-4.2, Supporting information**).

The adapted linkage-specific sialic acid neutralization resulted in ethyl esterification of α2,6-linked sialic acids and amidation of α2,3-linked sialic acids, by implementing an amidation step with ammonia directly after the original ethyl esterification incubation period. The end products remained intact during subsequent reducing end labeling at 60°C and compared to previously mentioned methods no intermediate cleanup was necessary.<sup>223,227</sup>

## PERMANENT CATIONIC LABELING OF THE *N*-GLYCAN REDUCING END

As both the non-sialylated and neutralized *N*-glycans do not carry a readily chargeable group, a chargeable group should be introduced to all glycoforms to enable CE separation. This is often achieved by labeling the reducing end of the *N*-glycans, for which reductive amination, using an arylamine, is the most common method.<sup>228,229</sup> After the reaction with the amine, reductive amination converts the Schiff-base into a stable secondary amine. Unfortunately, the resulting excess of reducing agent is usually not compatible with the subsequent analysis methods and a cleanup step is needed, inevitably inducing sample loss. The use of hydrazine or hydrazide labels has the advantage that no reduction (and subsequent clean up) is required, as the generated hydrazones are reasonably stable and have multiple resonance forms which lower their susceptibility to nucleophilic attacks.<sup>225,230</sup> Both hydrazine and hydrazide labels have proven their applicability in the analysis of released *N*-glycans by LC-ESI-MS and MALDI-TOF-MS methods, enhancing ionization and/or separation efficiency.<sup>225</sup> Here, we introduced



the hydrazide GirP, carrying a permanent positive charge, to all *N*-glycans after the linkage-specific neutralization of the sialic acids by either EEA (current study) or DA (**Figure 4.1.C**).<sup>222</sup> The optimization of the reducing end labeling with the hydrazide label started with reaction conditions based on literature,<sup>231-233</sup> as described in **Section S-4-1-5**, Supporting Information. However, instead of the commonly used methanol, we used EtOH as solvent, because of its lower toxicity. Labeling efficiency was evaluated based on MALDI-TOF-MS analysis of the HILIC SPE-purified reaction products, taking into account seven *N*-glycan signals covering di- and tri-antennary species with and without sialic acids (**Figure S-4.3, Supporting information**). Initial experiments revealed the labeling efficiency to be highly dependent on the vacuum concentrator used to dry the samples after the reducing end labeling (data not shown). To improve the robustness of the labeling, the reaction conditions were optimized in such a way that the labeling was already at its maximum before vacuum concentration. In the first optimization step, the fraction of water in the GirP reagent was reduced from 5% to 0% resulting in a labeling efficiency of  $52\% \pm 7\%$  (**Table S-4.1, Supporting information**). Reducing the water content was expected to push the equilibrium to completeness, as water is one of the reaction products. Subsequently, the GirP concentration was increased to 50 mM, resulting in a labeling efficiency of  $72\% \pm 4\%$  (**Table S-4.1, Supporting information**). Drying the *N*-glycans prior to addition of the reagent further reduced the water content of the final mixture. In this way we were able to reduce the reagent volume to 2  $\mu\text{L}$  (labeling efficiency  $89\% \pm 5\%$ ) (**Table S-4.1, Supporting information**). Finally, the incubation time was reduced to 1 h for practical reasons, resulting in a labeling efficiency of  $87\% \pm 4\%$  for the optimized protocol (**Table S-4.1, Supporting information**).

## CE-ESI-MS(/MS) OF GIRARD'S REAGENT P LABELED *N*-GLYCANS

### IMPLEMENTATION OF DOPANT ENRICHED NITROGEN GAS AT THE INTERFACE BETWEEN CE AND MS

Previously, an improved ESI efficiency for peptides and glycopeptides was obtained when a nebulizer, a dry gas enriched with MeCN, was infused into the ionization source (DEN-gas) for both (nano)LC-ESI-MS and CE-ESI-MS platforms.<sup>194,226</sup> Here, we evaluated the added value of using DEN-gas for the sensitivity of GirP labeled *N*-glycan detection with CE-ESI-MS. The incorporation of DEN-gas at the interface between the CE and the MS resulted in an overall lower background, particularly in the higher *m/z* region ( $m/z > 500$ ; **Figure S-4.4, Supporting information**). Furthermore, a higher intensity for the base peak electropherogram was obtained (**Figure S-4.4.A.2 and S-4.4.B.2, Supporting information**). The advantages of using DEN-gas were further supported by comparing the analysis of the 20 most abundant *N*-glycans with and without the use of DEN-gas (**Figure S-4.5, Supporting information**). While the relative abundances of the *N*-glycans remained the same (**Figure S-4.5.A, Supporting**

information), an average 3-fold increase in S/N (**Figure S-4.5.B, Supporting information**) and a 2-fold increase in absolute peak area (**Figure S-4.5.C, Supporting information**) were observed when using DEN-gas. Based on this, DEN-gas was employed for all further analyses.

## SENSITIVITY ASSESSMENT

The sensitivity of the CE-ESI-MS platform with DEN-gas was evaluated using two *N*-glycan standards in known amounts (H3N4 and H5N4, **Table S-4.2, Supporting information**). These standards were selected, as they have no likely isomeric structural variants, which would hamper an exact determination of the sensitivity. The sensitivity of the CE-ESI-MS platform was compared to that of a commonly used MALDI-TOF-MS platform for released *N*-glycan analysis. Starting with 10,000 fmol of both standards, a dilution range down to 1 fmol covered the limit of quantification for both the CE-ESI-MS and the MALDI-TOF-MS platform. A starting amount of 5 fmol of *N*-glycans was sufficient for detection and quantification by CE-ESI-MS, while for MALDI-TOF-MS starting amounts of 500 fmol were necessary (**Table 4.1**).

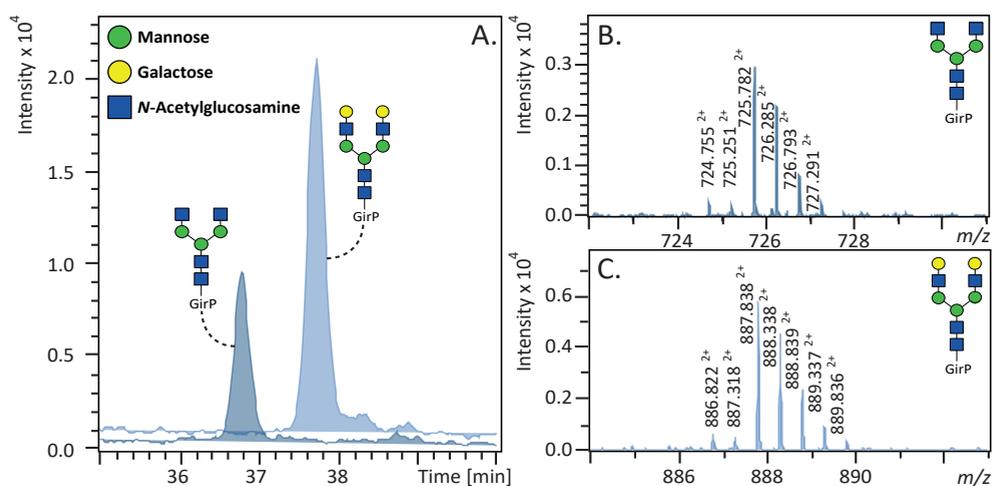
**Table 4.1: Sensitivity assessment of the H3N4 and H5N4 *N*-glycan standards as analyzed with MALDI-TOF-MS following EEA, or CE-ESI-MS with DEN-gas after additional labeling with GirP.** The presented values correspond to the lowest amounts of material measured, that still allowed reliable quantification ( $N = 3$ , **Supporting information, Table S-4.2**).

	Starting amount	Consumed molar amount	Average S/N $\pm$ SD	
			H3N4	H5N4
<b>CE-ESI-MS</b>	5 fmol	20 amol *	49 $\pm$ 18	146 $\pm$ 52
<b>MALDI-TOF-MS</b>	500 fmol	50 fmol	23 $\pm$ 8	45 $\pm$ 26

\* Corresponding to an injection volume of 43 nL (2  $\mu$ l sample vial)

The relative abundances of the two standards remained consistent between the different concentrations (down to the aforementioned minimum starting amounts) and platforms (**Figure S-4.6, Supporting information**). For the CE-ESI-MS platform, the 5 fmol starting amount corresponded to the injection of 20 amol (43 nL corresponding to 1/250 of the sample) of H3N4 and H5N4 with detection at S/N 49  $\pm$  18 and 146  $\pm$  52, respectively (**Figure 4.2, Table 4.1**). For MALDI-TOF-MS, 50 fmol of H3N4 and H5N4 was spotted on the target (1  $\mu$ L corresponding to 1/10 of the sample) with detection at S/N 23  $\pm$  8 and 45  $\pm$  26, respectively (**Table 4.1**). These results indicate the potential use of the presented CE-ESI-MS workflow for the analysis of samples for which ultrahigh-sensitivity is needed. We additionally envision that, for samples primarily analyzed by MALDI-TOF-MS after EEA, in-depth analysis can be performed by CE-ESI-MS with minimal sample consumption.





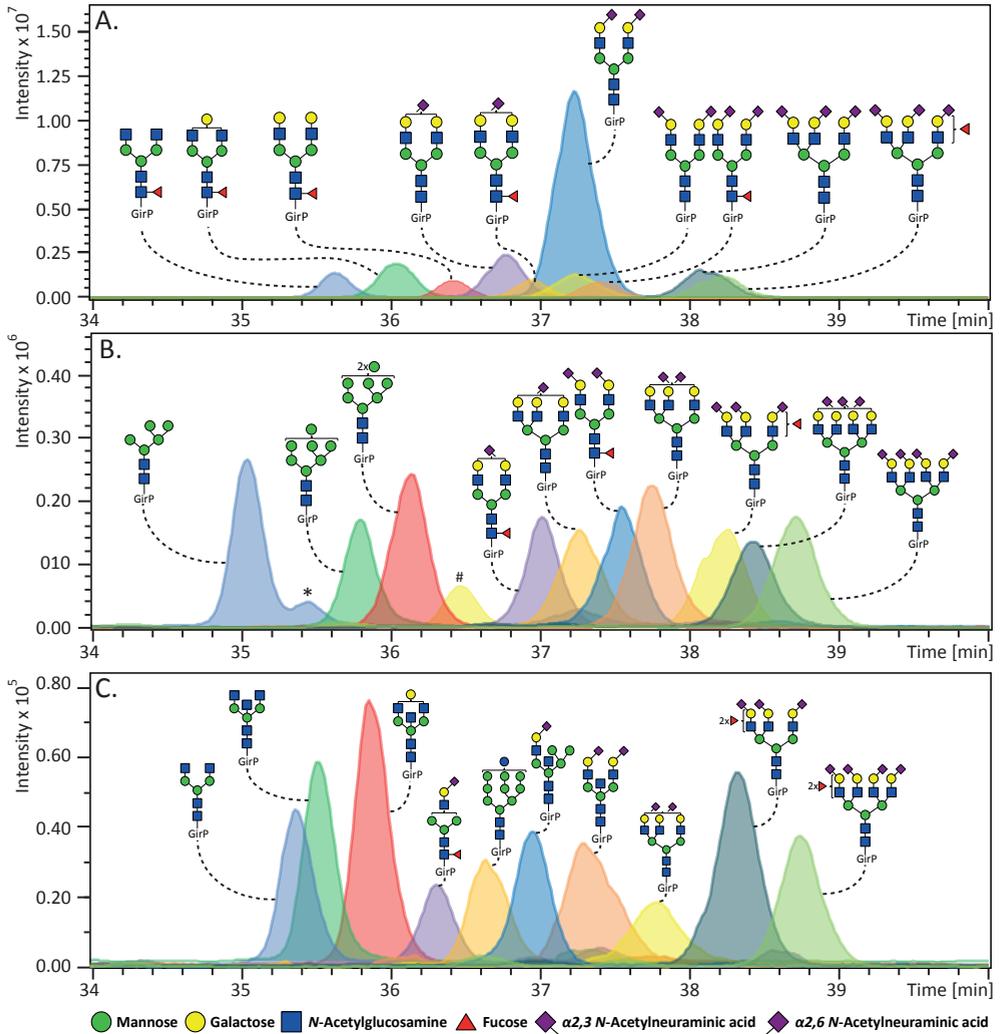
**Figure 4.2:** CE-ESI-MS analysis of the two *N*-glycan standards using DEN-gas, after sialic acid derivatization (EEA) and reducing end labeling with GirP on 5 fmol of both glycoforms. (A) Extracted ion electropherograms of H3N4 and H5N4 after injection of a final amount of 20 amol. Spectra of the doubly charged species corresponding to (B) H3N4 and (C) H5N4. GirP illustrates the Girard's reagent P label, attached to the glycans.

### IN-DEPTH ANALYSIS OF TPNG RELEASED *N*-GLYCANS

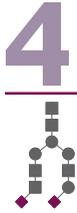
The CE-ESI-MS(/MS) platform for the analysis of GirP-labeled *N*-glycans was used to perform an in-depth investigation of the TPNG (Figure 4.3). Collision-induced dissociation (CID) MS/MS spectra were obtained for 82 unique *N*-glycan compositions revealing a broad range of structural features. Based on mass accuracy (< 10 ppm error) and migration behavior, an additional 85 *N*-glycans could be identified bringing the total number of detected *N*-glycans to 167. Table S-4.3.1 in the Supporting information (Excel file) provides an overview of the TPNG *N*-glycans found in the current study as compared to literature. An overview of the *N*-glycans that were not observed in this study but were observed in previous studies is given in Table S-4.3.2, Supporting information (Excel file). The observed discrepancies might be partially caused by biological variation between the analyzed samples.

Rather low amounts of *N*-glycans with underivatized sialic acids were found, as expected due to the stable derivatization of both  $\alpha$ 2,3- and  $\alpha$ 2,6-linked sialic acids implemented in our workflow.<sup>222,223</sup> The underivatized variants of the most abundant *N*-glycan (H5N4S<sub>2,6</sub>-2) and its isomeric variants (H5N4S<sub>2,6</sub>-1S<sub>2,3</sub>-1 and H5N4S<sub>2,3</sub>-2), only accounted for 1.8%  $\pm$  0.7% (H5N4S<sub>2,6</sub>-1S<sub>underivatized</sub>-1) and 0.1%  $\pm$  0.04% (H5N4S<sub>2,3</sub>-1S<sub>underivatized</sub>-1) of the total H5N4S2-related signals (Figure S-4.7, Supporting information). The H5N4S1-related signals reveal similarly high derivatization efficiencies, with the ethyl-esterified species dominating (H5N4S<sub>2,6</sub>-1; 94.0%  $\pm$  0.9%) followed by the amidated variant (H5N4S<sub>2,3</sub>-1; 5.1%  $\pm$  0.6%), and only 0.9%  $\pm$  0.03% of the area accounting for the underivatized sialic acid form (H5N4S<sub>underivatized</sub>-1). Altogether, the EEA protocol presented in this study has a high derivatization efficiency and

results in products that are stable over time and during subsequent processing at elevated temperatures. Furthermore, *N*-glycans without GirP-label, or sialylated species with more than one underivatized sialic acid, were not found in the CE-ESI-MS analysis. This may be partially due to the selectivity of the CE separation and ESI-MS detection, as we know that a minor fraction of the glycans was not reducing end-labeled, based on the MALDI-TOF-MS experiments (Table S-4.1, Supporting information).



**Figure 4.3:** Extracted ion electropherograms of released TPNG *N*-glycans after sialic acid derivatization (EEA) and reducing end labeling with GirP. **(A)** The 10 most abundant *N*-glycans (> 2% relative abundance), **(B)** 10 medium abundant *N*-glycans (between 0.5 - 1.0% relative abundance) and **(C)** 10 low abundant *N*-glycans (< 0.25% relative abundance) are displayed. Separation was achieved using a bare fused silica capillary after dynamic neutral coating. The CE-ESI-MS analysis was performed using DEN-gas. GirP illustrates the Girard's reagent P label, attached to the *N*-glycans. \* Indicates in-source decay of the Man6. # Shows the presence of doubly charged species of H5N5F1 of which the *m/z* values overlap with the ones of the triply charged species of H6N5F1<sub>2,3</sub>2S<sub>2,6</sub>1.



GirP carries a permanent positive charge on its quaternary amine group, enabling normal mode CE separation of labeled *N*-glycans under a broad range of conditions. Permanently charged *N*-glycans have shown before to result in a lower sensitivity during ESI than *N*-glycans with a more hydrophobic label, carrying a proton acceptor group.<sup>232,234</sup> However, a permanent charge at the *N*-glycan reducing end aids the fragmentation in MS/MS by providing permanently charged *Y*-ions.<sup>233</sup> Interestingly, some distinct diagnostic ions could be identified in the MS/MS data of the GirP-labeled *N*-glycans (**Figure S-4.8** and **Table S-4.3.3, Supporting information**). The *Y*-ion detected at  $m/z$  355.162<sup>1+</sup> corresponds to the GirP label with an *N*-acetylglucosamine, while the ion at  $m/z$  501.220<sup>1+</sup> indicates the presence of core-fucosylation; [GirP + *N*-acetylglucosamine + fucose]<sup>+</sup>. Bisection was identified by a *Y*-ion present at  $m/z$  923.373<sup>1+</sup>, or in combination with a core-fucose at  $m/z$  1069.431<sup>1+</sup>. In addition, several diagnostic oxonium ions (*B*-ions) were detected. The signal at  $m/z$  291.119<sup>1+</sup> corresponds to an amidated sialic acid ( $\alpha$ 2,3-linked) while  $m/z$  320.134<sup>1+</sup> corresponds to an ethyl-esterified sialic acid ( $\alpha$ 2,6-linked). The observed *B*-ion at  $m/z$  325.113<sup>1+</sup> corresponds to two hexoses, mainly observed in high mannose *N*-glycans. The signal at  $m/z$  366.140<sup>1+</sup> typically indicates an antenna [hexose + *N*-acetylhexosamine]<sup>+</sup>, observed for complex and hybrid-type *N*-glycans. While a signal at  $m/z$  512.197<sup>1+</sup> indicates a fucose attached to an antenna [hexose + *N*-acetylhexosamine + fucose]<sup>+</sup>. Signals at  $m/z$  656.251<sup>1+</sup> and  $m/z$  685.260<sup>1+</sup> are *B*-ions indicating a full antenna loss with an amidated ( $\alpha$ 2,3-linked) or an ethyl-esterified ( $\alpha$ 2,6-linked) sialic acid, respectively. In the case of a fucosylated,  $\alpha$ 2,3-linked sialylated antenna, a *B*-ion at  $m/z$  802.309<sup>1+</sup> was observed. Notably, no fucosylation was observed for antennae with ethyl-esterified ( $\alpha$ 2,6-linked) sialic acids, indicating that antenna fucosylation in TPNG occurs solely in the context of sialyl Lewis-type structures such as sialyl Lewis X.<sup>235,236</sup> Furthermore, MS/MS of fucosylated *N*-glycans with more than two antennae, generally indicated antenna fucosylation (except from when they were exclusively  $\alpha$ 2,6-sialylated). Mono- and diantennary *N*-glycans, on the other hand, showed mainly core-fucosylation.

### REPEATABILITY AND INTERMEDIATE PRECISION OF TPNG RELEASED *N*-GLYCAN ANALYSIS

The final CE-ESI-MS assay was evaluated for its repeatability and intermediate precision by preparing three separate samples per day on three consecutive days including the *N*-glycan release step, sialic acid derivatization (EEA) and reducing end labeling. Samples that underwent the same sample treatment except for the labeling procedure, were analyzed on a commonly used MALDI-TOF-MS platform. This allowed the comparison between the two analytical methods. The CE-ESI-MS platform detected 167 unique *N*-glycans, of which 118 *N*-glycans could be quantified (ppm error  $\pm$  10 ppm, S/N > 9 and isotopic pattern quality score (IPQ) < 20%; **Table S-4.3.1, Supporting information** (Excel file)). After total area normalization of the 118 glycoforms, the median relative standard deviation (RSD) of the 20 most abundant *N*-glycans (accounting for about 80% of the summed *N*-glycans

quantified in TPNG) was found to be 6.8% for the repeatability (intraday variability day 1), while the intermediate precision (interday variability day 1 to 3) resulted in a median RSD of 9.4% (**Figure S-4.9, Supporting information**). With the MALDI-TOF-MS platform, a total of 76 *N*-glycans were detected, of which 54 *N*-glycans were quantified (ppm error  $\pm$  20 ppm, S/N > 9 and IPQ < 20%; **Table S-4.3.1, Supporting information**). The median RSD of the 20 most abundant *N*-glycans was found to be 9.4% for the repeatability (intraday variability day 1) while the intermediate precision (interday variability) resulted in a median RSD of 15.1% (**Figure S-4.10, Supporting information**), which was slightly higher than the level reported before for the MALDI-TOF-MS platform.<sup>160,208</sup> The rather low RSDs obtained with the CE-ESI-MS method indicates that the extra sample preparation step required for the introduction of the cationic reducing end label hardly compromises the repeatability of the method. The higher variability of the MALDI-TOF-MS platform compared to the CE-ESI-MS platform is most likely introduced by the heterogeneous crystallization of the samples on the MALDI target. For the TPNG samples, the lowest abundant glycoform that could be reliably quantified using the MALDI-TOF-MS platform (H6N5F2S<sub>2,3</sub>1S<sub>2,6</sub>2) had a relative abundance of approximately 0.08%, while for the CE-ESI-MS platform this was about ten times lower at 0.007% (H8N7F1S<sub>2,3</sub>4), resulting in the coverage of a vaster range of *N*-glycan species.

## FUTURE PERSPECTIVES

The current work describes an ultrahigh-sensitive CE-ESI-MS platform for the analysis of released *N*-glycans. The complete and straightforward workflow involves sialic acid linkage-specific derivatization, purification of the *N*-glycans by HILIC SPE, reducing end labeling with the cationic GirP and, finally, separation and detection by CE-ESI-MS.

This study allowed a relatively rapid CE separation (5 min separation window) using a dynamic, neutral capillary coating and a constant pressure of 0.5 psi. Further optimization of the CE conditions would enable the separation of isomeric structures, on top of the separation of sialic acid linkage-isomers already achieved with the current method. This could lead to an even deeper insight into complex samples, like the TPNG. For example, the differentiation between arm galactosylation and sialylation, and the presence of either a bisecting *N*-acetylglucosamine or an extra antenna would be of interest. We foresee opportunities to further enhance the CE separation by using different capillary coatings, for example ones that would minimize the EOF. Additionally, the effect of the use of different background electrolytes, including different organic modifiers, pH and leading electrolytes should be evaluated for this purpose.<sup>237,238</sup>

It should be noted that GirP is a rather cost-efficient chemical compared to APTS which is commonly used for *N*-glycan labeling in CE and is comparable to 2-AB (2-aminobenzamide) often used in LC. The investigation of different hydrazide/hydrazine labels might improve



the separation efficiency and/or the sensitivity of the platform even further. For example, the use of a label with multiple charges (or chargeable groups under specific CE conditions), will likely improve the separation efficiency, while more hydrophobic labels will improve the ion-desolvation during the ESI process.<sup>234</sup> Therefore, depending on the exact requirements of an assay, further research should focus on the use of labels with different properties. Another interesting aspect would be the comparison between the presented CE-ESI-MS platform (based on normal mode CE) and well-established assays using negatively charged (or chargeable) labels (*e.g.*, APTS, ANTS or 2-aminobenzoic acid (2-AA)) on reversed mode CE-ESI-MS platforms with negative mode MS detection.<sup>216</sup> Based on previous research, we expect an overall lower sensitivity when labeled *N*-glycans are detected in negative ionization mode MS, as compared to positive ionization mode.<sup>239</sup> The main advantage of the presented CE-ESI-MS platform as compared to nano-LC-MS, is its ability to operate under very low flow rates (< 10 nL/min) which provides maximum sensitivity.<sup>199,226</sup> It would be of great interest to compare the latter ultrahigh-sensitivity glycoanalytical platforms to assess their differences in sensitivity as well as their complementarity, for example in terms of separation.

## CONCLUSION

In this work, an ultrahigh-sensitivity CE-ESI-MS(/MS) platform was presented for the sialic acid linkage-specific analysis of *N*-glycans with a cationic tag. This platform is highly complementary to more high-throughput MALDI-TOF-MS platforms, as the current CE-ESI-MS(/MS) method provides in-depth information, even on low abundant *N*-glycan species (relative abundance of 0.007%) in highly complex samples. The CE-ESI-MS platform can be used on volume restricted samples with rather low concentrations when conventional methods are not able to provide the desired sensitivity. For the analyzed TPNG sample, a total of 167 unique *N*-glycan compositions was identified, compared to 76 unique compositions detected for the same sample with a MALDI-TOF-MS platform. The CE-ESI-MS platform turned out to be 100 times more sensitive than the MALDI-TOF-MS method in terms of starting amounts used (5 fmol *versus* 500 fmol), and even 2500 times more sensitive regarding the absolute amounts analyzed (20 amol *versus* 50 fmol). We believe that the developed platform is of interest for thorough analysis of complex *N*-glycan samples as well as for samples that are too low in their concentration to be measured with conventional methods.

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## SUPPORTING INFORMATION

The full supplementary information of this thesis can be found via <https://doi.org/10.17026/dans-zg4-nxca>.



