Label-Free Analysis by UHPLC with Charged Aerosol Detection of Glycans Separated by Charge, Size, and Isomeric Structure

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Application Note 1127

Key Words
Glycan Quantitation, Glycan Profiling, Glycoprotein, Native Glycan, Universal Detection, Mixed-mode Separation

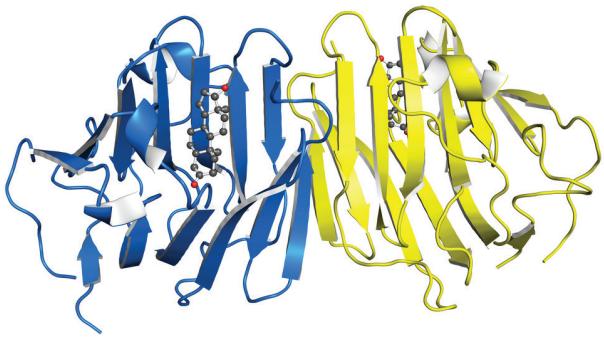
Goal

To develop fast and sensitive UHPLC methods suitable to directly measure the glycan content and profile of glycoproteins.

Introduction

Glycoproteins of biological, diagnostic, or therapeutic interest owe key aspects of their normal function to the oligosaccharides attached to the protein backbone. Changes in the number, type, composition, or linkage pattern of these glycans may serve as a biomarker of disease or influence the efficacy of a biotherapeutic product.[1] For this reason, the ability to correctly identify and measure these glycans is of scientific interest, and to do so reliably, quickly, and inexpensively is of practical benefit.

Glycans are polar molecules that are weakly retained on reversed-phase liquid chromatography columns but more strongly retained by porous graphitic carbon, HILIC, anion-exchange, or mixed-mode columns. Because N-linked glycans are not well-detected by UV/Vis absorption detectors, the separated glycans are detected directly by pulsed amperometry or mass spectrometry or by fluorescence after labeling with 2-AA or 2-AB. Nearly every combination of separation and detection mode has been demonstrated, but the most common combinations for separation and detection of glycans are HILIC-FLD[2] and HPAE-PAD[3] for quantitation, and HILIC-MS and mixed-mode-MS[4] for identification and characterization.



This application note describes direct detection of native glycans as an alternative to the common techniques for glycan analysis that rely on derivatization reactions to render glycans detectable. The lack of a detectable chromophore in native glycans is overcome by using HPLC with charged aerosol detection, a detector that can quantitatively measure any non-volatile compound.

N-linked glycans are released from proteins by PNGase-F. The native glycans are separated by ultra-high-performance liquid chromatography (UHPLC) on a column that employs both weak anion exchange and reversed-phase separation mechanisms, thus resolving glycans based on charge, isomeric structure, and size. As a result, this column supports direct glycan quantification of many more isoforms than can be quantified using other column types. The native glycans are detected directly without derivatization by using charged aerosol detection (CAD).

Experimental

Instrument

Thermo Scientific™ Vanquish™ UHPLC system with:

- System Base (P/N VH-S01-A)
- Vanquish Charged Aerosol Detector H (P/N VH-D20-A)
- Vanquish Binary Pump H (P/N VH-P10-A)
- Vanquish Column Compartment H (VH-C10-A)
- Vanquish Split Sampler HT (VH-A10-A)
- Vanquish Diode Array Detector HL (VH-D10-A)

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Consumables

- Ultrapure water (deionized, UV-treated, 0.2 µm filtered), 18.2 MΩ•cm, TOC ≤ 5 ppb
- Ammonium formate, ≥99%, Optima™ LC/MS (Fisher Scientific™ A115-50)
- Ammonium hydroxide, Optima (Fisher Scientific A470-250)
- Formic acid, ≥99.5%, Optima LC/MS (Fisher Scientific A117-50)
- Acetonitrile, ≥99.9%, Optima LC/MS (Fisher Scientific A955-4)
- Methanol, ≥99.9%, Optima LC/MS (Fisher Scientific A456-4)
- Thermo Scientific™ Dionex™ OligoStandard™ Sialylated Fetuin N-linked Alditols (043604)
- Sialylated glycan standards, mono-sialylated A1 (GKC-124300), di-sialylated A2 (GKC-224300), and tri-sialylated A3 (GKC-335300) (ProZyme®)
- α1-acid glycoprotein (orosomucoid), 99%, (Sigma-Aldrich® G3643)
- Fetuin from fetal bovine serum (MP Biomedicals, 02199810)
- PNGase F Deglycosylation kit (QA-Bio E-PNG01)
- Thermo Scientific Vial kit, 0.3 mL polypropylene with caps and septa (NC9870012)
- Thermo Scientific Snap cap low retention microcentrifuge tubes (3434ECONO)
- Analytical column, Thermo Scientific™ GlycanPac™ AXR-1 column, 1.9 µm, 2.1 × 100 mm (088136)

Mobile Phase Preparation

Because the charged aerosol detector will detect all non-volatile and many semi-volatile chemical species, including contaminants in the mobile phase and samples, use only high purity solvents and reagents, such as Fisher Optima LC/MS-grade. Ultrapure water used to prepare the mobile phase, standards, and samples must be 18.2-megohm-cm (Type 1 Reagent or HPLC Grade) water. It should be free of ionized impurities, organics, microorganisms, and particulate matter larger than 0.2 µm. Many commercial water purifiers are designed for HPLC applications and are suitable for this application.

Mobile phase B, 100 mM, pH 4.4 ammonium formate buffer

In a 1.00 L volumetric flask, dissolve 6.35 ± 0.05 g of ammonium formate in ultrapure water. Add 0.70 ± 0.05 g of formic acid. Adjust the pH to 4.4 if necessary by using dilute formic acid or ammonium hydroxide. Bring to volume with ultrapure water and thoroughly mix.

Sample Preparation

OligoStandard

Add 500 µL of ultrapure water to one vial of OligoStandard sialylated fetuin N-linked alditols. Vortex to dissolve and transfer to a plastic HPLC autosampler vial.

Glycan standards

Add 50 µL of ultrapure water to one vial (10 µg) of each glycan standard. Vortex to mix and transfer to a polypropylene HPLC autosampler vial.

Protein samples

Prepare alpha acid glycoprotein or fetuin by dissolving 4.0 mg ± 1 mg of the lyophilized protein in 1 mL ultrapure water.

Prepare protein PNGase F digestions by using PNGase F Deglycosylation kit or equivalent per the manufacturer's instructions. Include a matrix blank substituting ultrapure water for the protein solution each time samples are digested. Briefly, add 35 µL of protein solution to a plastic centrifuge tube. Add 10 µL 5x reaction buffer 7.5 and 2.5 µL of denaturation solution. Heat at 100 °C for 5 minutes. Cool to room temperature. Add 2.5 µL of Triton X-100 and mix. Add 2.0 µL of PNGase F to the reaction. Incubate 18 hours at 37 °C. Centrifuge at 6720 x g for 10 min and inject the supernatant.

Chromatographic Conditions	
Column:	GlycanPac AXR-1 column, 1.9 µm, 2.1 × 100 mm
Column temp:	30 °C
Flow rate:	0.4 mL/min
Injection volume:	2 µL
Mobile phase A:	Ultrapure deionized water
Mobile phase B:	100 mM ammonium formate pH 4.4
Gradient:	See Table 1
Detection:	Charged aerosol: • Evaporation temperature: 50 °C • Power function: 1.00 • Data collection rate: 10 Hz • Signal filter: 5 s

Table 1. Gradient.

Time (min)	Flow (mL/min)	%B
-8	0.400	4
0	0.400	4
35	0.400	39

Data Analysis

The Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System (CDS) 7.2 SR2 MUa was used for data analysis.

Results and Discussion

Method Development

Three method parameters were optimized during development of this UHPLC-CAD method*. First, from a starting concentration of 4 mM ammonium formate, the gradient slope was optimized by comparing glycan resolution and total run time for gradient slopes ranging from 0.5–3 mM/min. The optimum gradient slope was 1 mM/min, as seen in Figure 1, so this was chosen for the final method.

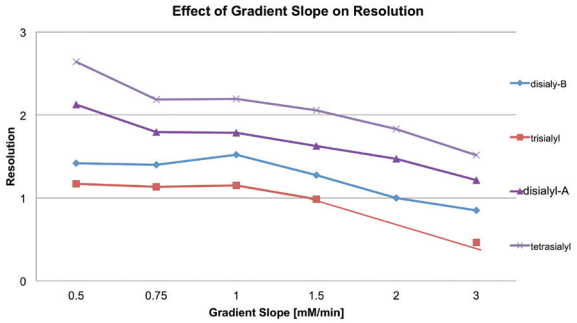

Figure 1. Optimizing the mobile phase gradient slope for analysis of native glycans by UHPLC-CAD on the GlycanPac AXR-1 column.

Secondly, mobile phase composition was optimized by examining the S/N of analytes separated with mobile phases containing from 0–20% acetonitrile or methanol. Although the signal increased with increasing organic solvent, the S/N ratio varied only slightly and peaked at 1% or 5% (Figure 2). Given the only modest increase in S/N provided by added solvent, we chose to use a purely aqueous gradient for simplicity and robustness.

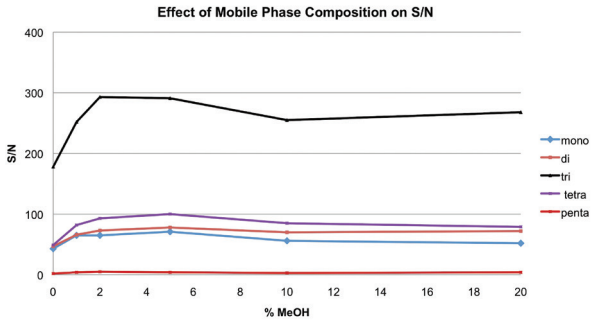

Figure 2. Optimizing the mobile phase composition for analysis of native glycans by UHPLC-CAD on the GlycanPac AXR-1 column.

Finally, the effect of evaporation tube temperature was considered by examining S/N for the glycan analytes at evaporation temperature settings of 35, 50, and 80 °C. Although S/N clearly decreased at 80 °C, the differences between 35 °C and 50 °C were less pronounced (Figure 3), and 50 °C was chosen as the evaporation temperature for the final method.

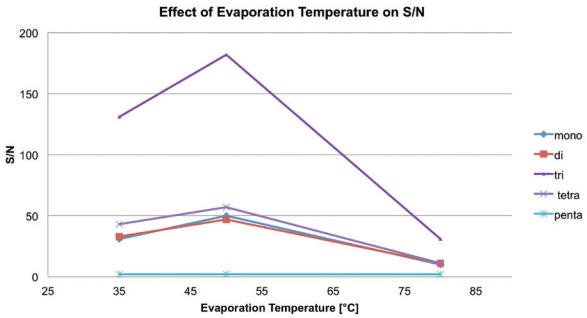

Figure 3. Optimizing the evaporation temperature for analysis of native glycans by UHPLC-CAD on the GlycanPac AXR-1 column.

High-Resolution Separation by Charge, Size, and Structure

Quality control labs profile a protein's glycan pool to assess lot-to-lot variability, stability/degradation, or level of impurities. In the UHPLC-CAD chromatogram shown in Figure 4, glycans in a standard mixture are separated according to charge, size, and isomeric structure. The native glycans are separated by using a binary gradient consisting of water and a volatile ammonium formate buffer and measured directly by using the Vanquish Charged Aerosol Detector H. The elution order is neutral glycans first, followed by glycans with a single negative charge (monosialylated), glycans with two negative charges (disialylated), and so on. The glycans comprising each charge group are separated by ion-exchange interactions. Within each well-separated charge group, glycans differing in size or isomeric structure are further resolved by reversed-phase interactions.

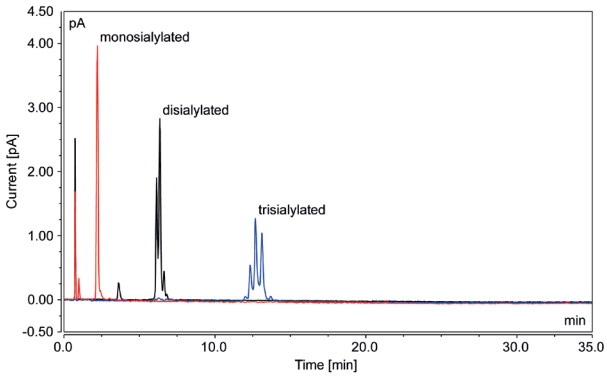

Figure 4. Direct charged aerosol detection of bovine fetuin N-linked alditols in a standard mixture separated with high-resolution on the GlycanPac AXR-1 column.

There is no need to use fluorescent labeling when using charged aerosol detection, as may be necessary with other means of detecting these compounds. Because of the uniform response of the charged aerosol detector, the relative peak area accurately reflects the amount (pmol) within this charge group. Note that under these conditions the neutral glycans are not well separated from the void peaks. To better resolve neutral glycans, use a shallower gradient, or derivatize the glycans to increase hydrophobicity (e.g., with 2-AB) or to introduce a negative charge (e.g., with 2-AA).

*(These initial experiments were performed on a different UHPLC system using a Thermo Scientific™ Dionex™ Corona™ Veo™ charged aerosol detector with performance similar to the Vanquish Charged Aerosol Detector H).

Performance

Calibration curves for native glycans containing one, two, and three sialic acid groups are presented in Figure 5. These native glycans are not identical to the reduced alditols present in the OligoStandard (bovine fetuin N-linked alditol standard), but are appropriate to evaluate method performance, and to quantify the sialylated N-linked glycans released from glycoproteins by PNGase F. Standards were prepared at concentrations ranging from 1.0 to 200 pmol/µL and analyzed in triplicate. The mass injected ranged from 2 to 400 pmol when using 2 µL injections. The peak area data were fitted with a quadratic equation, yielding coefficients of determination, R^2, greater than 0.995 for all three analytes. Table 2 presents a summary of the method's performance, including the coefficients of determination, limits of detection, and precision for glycan peaks corresponding to the three major charge groups of the sialylated fetuin N-linked alditol standard.

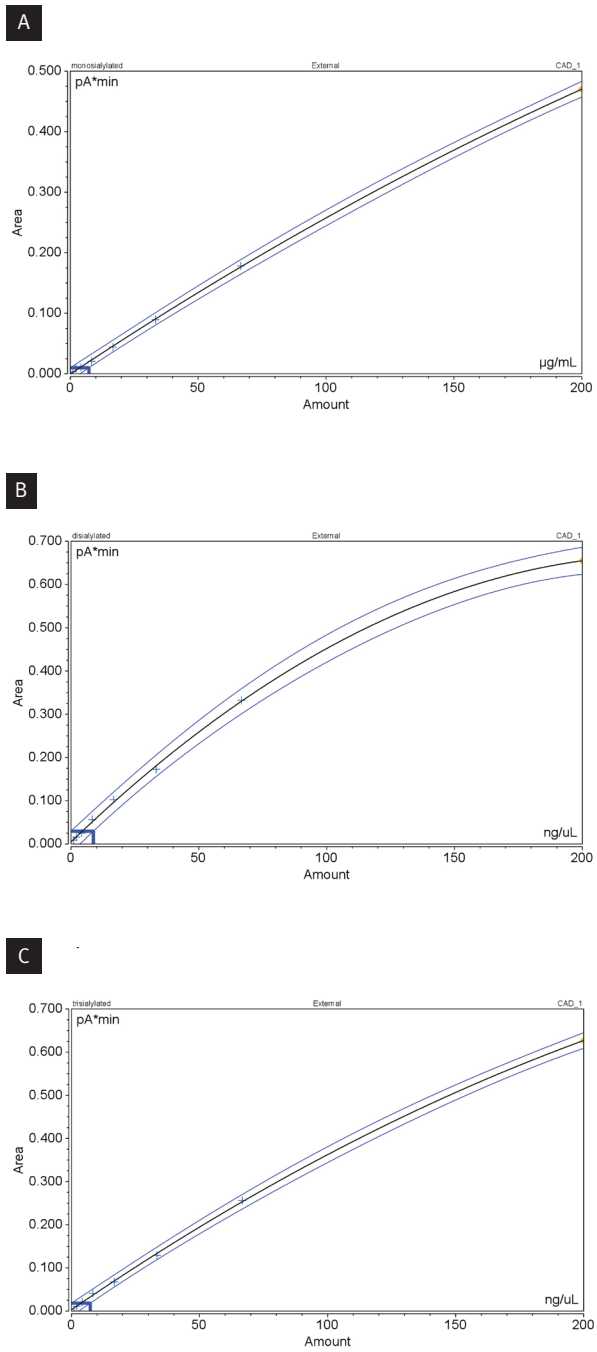

Figure 5. Calibration data for direct analysis of native N-linked glycans by UHPLC with charged aerosol detection on the GlycanPac AXR-1 column; A), monosialylated glycan standard; B), disialylated glycan standard; C), trisialylated glycan standard.

Table 2. Method performance for direct detection of glycans by HPLC-charged aerosol detection.

Concentration	Amount (ng/µL)	Amount[1] (pmol/uL)	Ret. Time[2] (%RSD)	Peak Area[2] (%RSD)	LOD[3] (ng/µL)	R²*
Monosialylated	12	6.1	0.11	2.7	7.3	0.999
Disialylated	22	10	0.04	1.2	8.8	0.997
Trisialylated	115	40	0.03	2.8	7.4	0.995
Tetrasialylated	19**	5.0	0.03	1.7	--	--

[1] Estimated from nominal MW of the glycan calibration standards
[2] For n = 7 replicates
[3] Hubaux-Vos method
* Eight levels, in triplicate (duplicate for highest concentration), quadratic fit with no offset
** Estimated from the peak area response factor of the disialylated glycan standard

Protein Digests

Charged aerosol detection is clearly highly sensitive and able to detect glycans at the low picomole concentration level. Because charged aerosol detection is universal, there might be concern that the reagents and reaction products remaining after endoglycosidase treatment would interfere with detection of the released glycans. To test for such interference, we treated two proteins by using a commercial glycan release kit and then analyzed for glycans by using UHPLC-CAD. Figure 6 shows that although additional peaks related to the reaction procedure are evident, they are well resolved from the glycan analytes and do not interfere with reliable quantification of the glycans. This demonstrates that where ultimate sensitivity is not required[3], UHPLC-CAD obviates the need to spend time or money on 2-AB labeling and delivers a clean chromatogram with no concern for reaction side products.

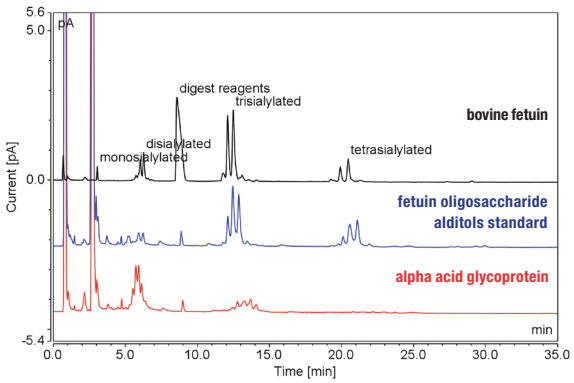

Figure 6. Direct charged aerosol detection of α1-acid glycoprotein and bovine fetuin N-linked glycans released by PNGase F and separated with high-resolution on the GlycanPac AXR-1 column.

Conclusion

Charged aerosol detection enables sensitive, direct measurement of glycans with no need to perform labeling reactions. Detection limits for native glycans are in the low pmol (ng on-column) range. The UHPLC-CAD method developed to measure native glycans is precise, with retention time precision better than 0.1% RSD and peak area precision averaging 2.8 % RSD for the major sialylated N-glycans of bovine fetuin. UHPLC-CAD allows accurate quantification of glycans with appropriate standards, and by responding similarly to all non-volatile analytes, yields simple, accurate and precise estimates of relative concentration even in the absence of pure primary standards.

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